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(54) Title: A RECOMBINANT ENZYME WITH MUTANASE ACTIVITY

(57) Abstract

The present invention relates to method for constructing an expression vector comprising a mutanase gene obtained from a filamentous fungus suitable for heterologous production comprising the steps of a) isolating a DNA sequence encoding a mutanase from a filamentous fungus, b) introducing a kex2-site or kex2-like site between the DNA sequences encoding the pro-peptide and the mature region of the mutanase, c) cloning the DNA sequence obtained in step b) into a suitable expression vector. The invention also relates to a recombinant expression vector comprising said mutanase gene sequence and a kex2 cleavage site between the DNA sequence encoding the pro-peptide and the region encoding the mature mutanase, a filamentous fungus host cell, a process for producing recombinant mutanase and a recombinant mutanase. It is also the object of the invention to provide compositions useful in oral care products for humans and animals.

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Title: A recombinant enzyme with mutanase activity

FIELD OF THE INVENTION

The present invention relates to a method for constructing an expression vector comprising a mutanase gene obtained from a filamentous fungus suitable for heterologous production, a recombinant expression vector comprising said mutanase gene sequence and a kex2 cleavage site between the DNA sequence encoding the pro-peptide and the DNA sequence encoding the mature mutanase, a filamentous fungus host cell, a process of producing recombinant mutanase, and said recombinant mutanase.

It is also the object of the invention to provide compositions useful in oral care products for humans and animals.

BACKGROUND OF THE INVENTION

Mutanases are α-1,3-glucanases (also known as α-1,3-glucanohydrolases) which degrade the α-1,3-glycosidic linkages in mutan. Mutanases have been described from two species of *Trichoderma* (Hasegawa et al., (1969), Journal of Biological Chemistry 244, p. 5460-5470; Guggenheim and Haller, (1972), Journal of Dental Research 51, p. 394-402) and from a strain of *Streptomyces* (Takehara et al., (1981), Journal of Bacteriology 145, p. 729-735), *Cladosporium resinae* (Hare et al. (1978), Carbohydrate Research 66, p. 245-264), *Pseudomonas* sp. (US patent no. 4,438,093), *Flavobacterium* sp. (JP 77038113), *Bacillus circulanse* (JP 63301788) and *Aspergillus* sp.. A mutanase gene from *Trichoderma harzianum* has been cloned and sequenced (Japanese Patent No. 4-58889-A from Nissin Shokuhin Kaisha LDT).

Although mutanases have commercial potential for use as an antiplaque agent in dental applications and personal care products, *e.g.*, toothpaste, chewing gum, or other oral and dental care products, the art has been unable to produce mutanases in significant quantities to be commercial useful.

US patent no. 4,353,891 (Guggenheim et al.) concerns plaque removal using mutanase produced by *Trichoderma harzianum* CBS 243.71 to degrade mutan synthesized by cultivating *Streptococcus*

mutans strain CBS 350.71 identifiable as OMZ 176.

It is an object of the present invention to provide a recombinant mutanase from *Trichoderma harzianum* which can be produced in commercially useful quantities.

BRIEF DESCRIPTION OF THE DRAWING

Figure 1 shows plasmid pMT1796

Figure 2 shows plasmid construction of plasmids pMT1796, pMT1802, and pMT1815,

Figure 3 shows an outline of the construction of the A. oryzae recombinant mutanase expression vector pMT1802,

Figure 4 shows the pH-profile of recombinant and wild-*type T. harzianum* CBS 243.71 mutanase

Figure 5 shows the temperature profile of recombinant and wild-type *T. harzianum* CBS 243.71 mutanase at pH 7,

Figure 6 shows the temperature stability of recombinant and wild-type *T. harzianum* CBS 243.71 mutanase at pH 7,

Figure 7 shows the indirect Malthus standard curve for a mix culture of *S. mutans*, *A. viscosus* and *F. nucleatum* grown in BHI at 37°C.

SUMMARY OF THE INVENTION

The object of the invention is to provide a recombinant mutanase derived from a filamentous fungus by heterologous expression.

The present inventors have as the first been able to express the mutanase gene of a filamentous fungus heterologously and thus cleared the way for providing a single component, recombinant mutanase essentially free of any contaminants.

In the first aspect the invention relates to a method for constructing an expression vector comprising a mutanase gene obtained from a filamentous fungus suitable for heterologous production comprising the steps of:

a) isolating a DNA sequence encoding a mutanase from a filamentous fungus,

b) introducing a kex2 site or kex2-like site between the DNA sequences encoding the pro-peptide and the mature region of the

mutanase, or replacing the mutanase (pre)pro-sequence with a (pre)pro-sequence comprising a kex2 or kex2-like site of another fungal enzyme,

c) cloning the DNA sequence obtained in step b) into a suitable expression vector.

In a preferred embodiment the mutanase is obtained from a strain within the genus *Trichoderma*.

In step b) the mutanase (pre)pro-sequence may for instance be replaced with the Lipolase® (pre)pro-sequence or the TAKA-amylase (pre)pro-sequence.

It is also an object of the invention to provide an expression vector comprising a mutanase gene and a DNA sequence encoding a (pre)pro-peptide with a kex2 site or kex2-like site between the DNA sequences encoding said (pre)pro-peptide and the mature region of the mutanase.

The invention also relates to a filamentous host cell for production of recombinant mutanase derived from a filamentous fungus. Preferred host cells include filamentous fungi of the genera *Trichoderma*, *Aspergillus*, and *Fusarium*.

Further, the invention relates to a process for producing a recombinant mutanase in a host cell, comprising the steps:

a) transforming an expression vector comprising a mutanase gene with a kex2 site or kex2-like site between the DNA sequences encoding the pro-peptide and the mature region of the mutanase into a suitable filamentous fungus host cell,

b) cultivating the host cell in a suitable culture medium under conditions permitting expression and secretion of an active mutanase,

c) recovering and optionally purifying the secreted active recombinant mutanase from the culture medium.

The expression vector may be prepared according to the above described method of the invention.

A recombinant mutanase may according to the invention be produced according to the process of the invention.

A substantially pure wild-type mutanase obtained from *Trichoderma harzianum* CBS 243.71 essentially free of any contaminants is also part of the invention.

The invention also relates to a composition comprising a recombinant mutanase of the invention or a substantially pure mutanase of the invention useful in oral care products and food, feed and/or pet food products.

Finally the invention relates to the use of the recombinant mutanase of the invention or the substantially purified mutanase of the invention or composition or product of the invention preventing the formation of human or animal dental plaque or removing dental plaque and for the use in food, feed and/or pet food products.

DETAILED DESCRIPTION OF THE INVENTION

The object of the invention is to provide a recombinant mutanase derived from a filamentous fungus by heterologous expression.

The present inventors have as the first been able to express the mutanase gene of a filamentous fungus heterologously and thus cleared the way for providing a single component recombinant mutanase essentially free of any contaminants.

The principle of the invention can be used for all mutanases derivable from filamentous fungi, such as from filamentous fungi of the genus *Trichoderma*, such a strain of *Trichoderma harzianum*, especially *Trichoderma harzianum* CBS 243.71, *and* the genera *Streptomyces*, *Cladosporium or Aspergillus*.

Previously it has not been possible to produce mutanases of filamentous fungi heterologously. Consequently, according to prior art mutanases are produced homologously and comprise a mixture of other enzyme activities besides the mutanase (*i.e.* with undesired contaminants).

An example of this is *Trichoderma harzianum* CBS 243.71 which are known to produce a mutanase as also described above. The mutanase derived from *Trichoderma harzianum* CBS 243.71 has before the successful findings of the present invention only been produced homologously.

It is advantageous to be able to produce the mutanase heterologously, as it is then possible to provide a single component mutanase free of undesired contaminants. Further, it

facilitates providing an isolated and purified enzyme of the invention in industrial scale.

According to the invention it is possible to express mutanases derived from filamentous fungi in a suitable host cell by introducing a kex2 cleavage site or kex2-like site between the DNA sequences encoding the pro-peptide and the mature mutanase, or replacing the mutanase (pre)pro-sequence with a (pre)pro-sequence comprising a kex2 site or kex2-like site of another fungal enzyme.

The (pre)pro-sequence have for instance be the Lipolase® (pre)pro-sequence or the TAKA-amylase (pre)pro-sequence.

Pro-peptides

A large number of mature proteins are initially synthesised with a N-terminal extension, the pro-peptide, varying from very small peptides (*e.g.* GLA 6 amino acids) to relatively long peptides (*e.g.* PEPA 49 amino acids).

The pro-peptide can perform a number of different functions. Firstly, pro-peptides might contribute to the efficiency of co-translational translocation of the protein across the ER-membrane. Secondly, pro-peptides might contribute to co-translational proteolytic processing of the polypeptide. Thirdly, they might act as intracellular targeting signal for routing to specific cellular compartments. Fourthly, in some pro-proteins the pro-peptide keeps the protein inactive until it reaches its site of action.

Removal of the pro-peptide from the mature protein occurs in general by processing by a specific endopeptidase, usually after the two positively charged amino acid residues Arg-Arg, Arg-Lys or Lys-Arg. However, also other amino acid combinations, containing at least one basic amino acid, have been found to be processed.

The absence of these doublets in mature, endogenous secreted proteins might protect them from proteolytic cleavage. As di-basic cleavage is thought to occur in the Golgi, the internal di-basic peptide sequences in cytoplasmic proteins will not be attacked by this processing.

Kex2 sites

Kex2 sites (see *e.g.* Methods in Enzymology Vol 185, ed. D. Goeddel, Academic Press Inc. (1990), San Diego, CA, "Gene Expression Technology") and kex2-like sites are di-basic recognition sites (*i.e.* cleavage sites) found between the pro-peptide encoding region and the mature region of some proteins.

Insertion of a kex2 site or a kex2-like site have in certain cases been shown to improve correct endopeptidase processing at the pro-peptide cleavage site resulting in increased protein secretion levels.

However, in a number of other cases insertion of a Kex2 cleavage site did not increase the secretion level. For instance, Cullen et al., (1987), Bio/Technology, vol. 5, p. 369-376, found that insertion of a kex2 site in the secretion signal of chymosin (*i.e.* signal peptide and pro-peptide), which encoded the glucoamylase signal peptide and pro-peptide fused to prochymosin, did not increase the secretion level of recombinant chymosin expressed in a *Aspergillus nidulans* host cell.

Other examples of references showing that insertion of a kex2 site or a kex2-like site do not always increase the secretion level include Valverde et al., (1995), J. of Biolog. Chem, p. 15821-15826)

In the context of the present invention the term "heterologous" production means expression of a recombinant enzyme in an host organism different from the original donor organism or expression of a recombinant enzyme by the donor organism.

The term "homologous" production means expression of the wild-type enzyme by the original organism.

In the first aspect the invention relates to a method for construction of an expression vector comprising a mutanase gene suitable for heterologous production comprising the steps of:

a) isolating a DNA sequence encoding a mutanase from a filamentous fungus known to produce a mutanase,

b) introducing a kex2 site or kex2-like site between the DNA sequences encoding the pro-peptide and the mature region of the mutanase, or replacing the mutanase (pre)pro-sequence with a (pre)pro-sequence comprising a kex2 or kex2-like site of another fungal enzyme,

c) cloning the mutanase gene with the kex2 site or kex2-like site obtained in step b) into a suitable expression vector.

In a preferred embodiment of the mutanase gene is obtained from the genus *Trichoderma*, preferably a strain of the species *T. harzianum*, especially the strain *T. harzianum* CBS 243.71.

The complete mutanase gene DNA sequence derived from *Trichoderma harzianum* CBS 243.71 is shown in SEQ ID No. 1

In step b) the mutanase (pre)pro-sequence may for instance be replaced with the Lipolase® (pre)pro-sequence or the TAKA-amylase (pre)pro-sequence.

In the examples below illustrating the present invention a kex2-site is inserted into the *Trichoderma harzianum* mutanase gene presented in SEQ ID No. 1 as the site specific mutation E36 → K36.

Isolation of the mutanase gene

The DNA sequence encoding a mutanase may, in accordance with well-known procedures, conveniently be isolated from DNA from a suitable source, such as any of the above mentioned organisms known to comprise a mutanase gene, by use of synthetic oligo-nucleotide probes prepared on the basis of the DNA sequence disclosed herein.

For instance, a suitable oligonucleotide probe may be pre-pared on the basis of the nucleotide sequences shown in SEQ ID no. 1 or the amino acid sequence shown in SEQ ID no. 2 or any suitable sub-sequence thereof.

According to this method primers are designed from the knowledge to at least a part of SEQ ID No. 2. Fragments of mutanase gene are then PCR amplified by the use of these primers. These fragments are used as probes for cloning the complete gene.

Alternatively, the DNA sequence encoding a mutanase may be isolated by a general method involving

- cloning, in suitable vectors, a DNA or cDNA library from a strain of genus *Trichoderma*,
- transforming suitable host cells with said vectors,
- culturing the host cells under suitable conditions to express any enzyme of interest encoded by a clone in the DNA library,
- screening for positive clones by determining any mutanase activity of the enzyme produced by such clones, and
- isolating the DNA coding an enzyme from such clones.

The general method is further disclosed in WO 93/11249 the contents of which are hereby incorporated by reference.

Expression vector

In another aspect the invention relates to an expression vector comprising a mutanase gene and a DNA sequence encoding a pro-peptide with a kex2 site or kex2-like site inserted between the DNA sequences encoding said pro-peptide and the mature region of the mutanase.

In preferred embodiments of the invention the expression vector comprises besides the kex2 site or kex2-like site an operably linked DNA sequence encoding a prepro-peptide (*i.e.* signal peptide and a pro-peptide). The prepro-sequence may advantageously be the original mutanase signal-sequence or the Lipolase® signal-sequence or the TAKA signal-sequence and the original mutanase pro-sequence or the Lipolase® pro-sequence or the TAKA pro-sequence.

The promoter may be the TAKA promoter or the TAKA:TPI promoter.

In a specific embodiment of the invention the expression vector is the pMT1796 used to illustrate the concept of the invention in Example 3 below.

The choice of vector will often depend on the host cell into which it is to be introduced.

Thus, the vector may be an autonomously replicating vector, *i.e.* a vector which exists as an extrachromosomal entity, the

replication of which is independent of chromosomal replication, *e.g.* a plasmid. Alternatively, the vector may be one which, when introduced into a host cell, is integrated into the host cell genome and replicated together with the chromosome(s) into which it has been integrated.

In the vector, the DNA sequence encoding the mutanase should also be operably connected to a suitable promoter and terminator sequence. The promoter may be any DNA sequence which shows transcriptional activity in the host cell of choice and may be derived from genes encoding proteins either homologous or heterologous to the host cell.

The procedures used to ligate the DNA sequences coding for the mutanase, a prepro-sequence including the kex2 site or kex2-like site, the promoter and the terminator, respectively, and to insert them into suitable vectors are well known to persons skilled in the art (cf., for instance, Sambrook et al., (1989), Molecular Cloning. A Laboratory Manual, Cold Spring Harbor, NY).

Host Cell

A third aspect of the invention relates to a filamentous fungi host cell for production of recombinant mutanase derived from a filamentous fungus of the genus *Trichoderma*, such as a strain of *T. harzianum*, especially *T. harzianum* CBS 243.71, or the genus *Aspergillus*, such as a strain of *A. oryzae* or *A. niger*, or a strain of the genus *Fusarium*, such as a strain of *Fusarium oxysporium*, *Fusarium graminearum* (in the perfect state named *Gribberella zeae*, previously *Sphaeria zeae*, *synonym with Gibberella roseum and Gibberella roseum* f. sp. *cerealis)*, or *Fusarium sulphureum* (in the prefect state named *Gibberella puricaris*, synonym with *Fusarium trichothecioides*, *Fusarium bactridioides*, *Fusarium sambucium*, *Fusarium roseum*, and *Fusarium roseum var. graminearum)*, *Fusarium cerealis* (synonym with *Fusarium crokkwellnse)* or *Fusarium venenatum*.

The host cell may advantageously be a *F. graminearum* described in WO 96/00787 (from Novo Nordisk A/S), *e.g.* the strain deposited as *Fusarium graminearum* ATCC 20334. The strain ATCC

20334 was previously wrongly classified as *Fusarium graminearum* (Yoder, W. and Christianson, L. 1997). RAPD-based and classical taxonomic analyses have now revealed that the true identity of the Quorn fungus, ATCC 20334, is *Fusarium venenatum* Nirenburg sp. nov.

In a preferred embodiment of the invention the host cell is a protease deficient or protease minus strain.

This may for instance be the protease deficient strain *Aspergillus oryzae* JaL125 having the alkaline protease gene named "alp" deleted. This strain is described in PCT/DK97/00135 (from Novo Nordisk A/S).

Filamentous fungi cells may be transformed by a process involving protoplast formation and transformation of the protoplasts followed by regeneration of the cell wall in a manner known per se. The use of *Aspergillus* as a host microorganism is described in EP 238 023 (Novo Nordisk A/S), the contents of which are hereby incorporated by reference.

According to a further aspect the invention relates to a process for producing a recombinant mutanase in a host cell. Said process comprises the following steps:

a) transforming an expression vector encoding a mutanase gene with a kex2 site or a kex2-like site between the DNA sequences encoding the pro-peptide and the mature region of the mutanase into a suitable filamentous fungus host cell,

b) cultivating the host cell in a suitable culture medium under conditions permitting the expression of the expression vector,

c) recovering the secreted recombinant mutanase from the culture medium,

d) and optionally purifying the recombinant mutanase.

The recombinant expression vector may advantageously be any of the above described.

Further, the filamentous fungi host cells to be used for production of the recombinant mutanase of the invention according to the process of the invention may be any of the above mentioned host cell, especially of the genera *Aspergillus*, *Fusarium* or *Trichoderma*.

The medium used to culture the transformed host cells may be any conventional medium suitable for growing the host cells in question. The expressed mutanase is secreted into the culture medium and may be recovered from there by well-known procedures including separating the cells from the medium by centrifugation or filtration, precipitating proteinaceous components of the medium by means of a salt such as ammonium sulphate, followed by chromatographic procedures such as ion exchange chromatography, affinity chromatography, or the like.

It is also an important object of the invention to provide a recombinant mutanase produced according to the process of the invention.

The isolated recombinant mutanase has essentially an amino acid sequence as shown in SEQ ID no. 2. From SDS-PAGE a molecular weight around 80 kDa was found.

The pH optimum of the recombinant mutanase was found to lie in the range from 3.5 to 5.5 which equals the pH optimum of the wild-type mutanase (see Figure 4). The temperature optimum of both the recombinant and wild-type mutanase was found to be around 45°C at pH 7 and around 55°C at pH 5.5 (see Figure 5). Further, the residual activity starts to decline at 40°C at pH 7, while the enzyme is more stable at pH 5.5, where the residual activity starts to decline at 55°C.

The inventors have also provided a substantially pure wild-type mutanase obtained from *Trichoderma harzianum* CBS 243.71 essentially free of any active contaminants, such as other enzyme activities.

Composition

It is also an object of the invention to provide a composition comprising the recombinant mutanase of the invention or the purified wild-type mutanase essentially free of any active contaminants of the invention.

Oral care composition

In a still further aspect, the present invention relates to an oral care composition useful as an ingredient in oral care products.

An oral care composition of the invention may suitably comprise an amount of the recombinant *Trichoderma harzianum* mutanase equivalent to an enzyme activity, calculated as enzyme activity units in the final oral care product, in the range from 0.001 MU to 1000 MU/ml, preferably from 0.01 MU/ml to 500 MU/ml, such as from 0.1 MU/ml to 100 MU/ml, especially 0.05 MU/ml to 100 MU/ml.

It is also contemplated according to the invention to include other enzyme activities than mutanase activity in the oral care composition. Contemplated enzyme activities include activities from the group of enzymes comprising dextranases, oxidases, such as glucose oxidase, L-amino acid oxidase, peroxidases, such as *e.g.* the *Coprinus sp.* peroxidases described in WO 95/10602 (from Novo Nordisk A/S) or lactoperoxidaseor, haloperoxidases, laccases, proteases, such as papain, acidic protease (*e.g.* the acidic proteases described in WO 95/02044 (Novo Nordisk A/S)), endoglucosidases, lipases, amylases, including amyloglucosidases, such as AMG (from Novo Nordisk A/S), and mixtures thereof.

Oral care products

The oral care product may have any suitable physical form (*i.e.* powder, paste, gel, liquid, ointment, tablet etc.). An "oral care product" can be defined as a product which can be used for maintaining or improving the oral hygiene in the mouth of humans and animals, by preventing dental caries, preventing the formation of dental plaque and tartar, removing dental plaque and tartar, preventing and/or treating dental diseases etc.

At least in the context of the present invention oral care products do also encompass products for cleaning dentures, artificial teeth and the like.

Examples of such oral care products include toothpaste, dental cream, gel or tooth powder, odontic, mouth washes, pre- or post brushing rinse formulations, chewing gum, lozenges, and candy.

Toothpastes and tooth gels typically include abrasive polishing materials, foaming agents, flavouring agents, humectants, binders, thickeners, sweetening agents, whitening/bleaching/ stain removing agents, water, and optionally enzymes.

Mouth washes, including plaque removing liquids, typically comprise a water/alcohol solution, flavour, humectant, sweetener, foaming agent, colorant, and optionally enzymes.

Abrasives

Abrasive polishing material might also be incorporated into the dentifrice product of the invention. According to the invention said abrasive polishing material includes alumina and hydrates thereof, such as alpha alumina trihydrate, magnesium trisilicate, magnesium carbonate, kaolin, aluminosilicates, such as calcined aluminum silicate and aluminum silicate, calcium carbonate, zirconium silicate, and also powdered plastics, such as polyvinyl chloride, polyamides, polymethyl methacrylate, polystyrene, phenol-formaldehyde resins, melamine-formaldehyde resins, urea-formaldehyde resins, epoxy resins, powdered polyethylene, silica xerogels, hydrogels and aerogels and the like. Also suitable as abrasive agents are calcium pyrophosphate, water-insoluble alkali metaphosphates, dicalcium phosphate and/or its dihydrate, dicalcium orthophosphate, tricalcium phosphate, particulate hydroxyapatite and the like. It is also possible to employ mixtures of these substances.

Dependent on the oral care product the abrasive product may be present in from 0 to 70% by weight, preferably from 1% to 70%. For toothpastes the abrasive material content typically lies in the range of from 10% to 70% by weight of the final toothpaste product.

Humectants are employed to prevent loss of water from *e.g.* toothpastes. Suitable humectants for use in oral care products according to the invention include the following compounds and mixtures thereof: glycerol, polyol, sorbitol, polyethylene glycols (PEG), propylene glycol, 1,3-propanediol, 1,4-butanediol, hydrogenated partially hydrolysed polysaccharides and the like.

Humectants are in general present in from 0% to 80%, preferably 5 to 70% by weight in toothpaste.

Silica, starch, tragacanth gum, xanthan gum, extracts of Irish moss, alginates, pectin, cellulose derivatives, such as hydroxyethyl cellulose, sodium carboxymethyl cellulose and hydroxypropyl cellulose, polyacrylic acid and its salts, polyvinylpyrrolidone, can be mentioned as examples of suitable thickeners and binders, which helps stabilizing the dentifrice product. Thickeners may be present in toothpaste creams and gels in an amount of from 0.1 to 20% by weight, and binders to the extent of from 0.01 to 10% by weight of the final product.

Foaming agents

As foaming agent soap, an-ionic, cat-ionic, non-ionic, amphoteric and/or zwitterionic surfactants can be used. These may be present at levels of from 0% to 15%, preferably from 0.1 to 13%, more preferably from 0.25 to 10% by weight of the final product.

Surfactants

Surfactants are only suitable to the extent that they do not exert an inactivation effect on the present enzymes. Surfactants include fatty alcohol sulphates, salts of sulphonated monoglycerides or fatty acids having 10 to 20 carbon atoms, fatty acid-albumen condensation products, salts of fatty acids amides and taurines and/or salts of fatty acid esters of isethionic acid.

Sweetening agents

Suitable sweeteners include saccharin.

Flavouring agents

Flavours, such as spearmint, are usually present in low amounts, such as from 0.01% to about 5% by weight, especially from 0.1% to 5%.

Whitening/bleaching agents

Whitening/bleaching agents include H_2O_2 and may be added in amounts less that 5%, preferably from 0.25 to 4%, calculated on the basis of the weight of the final product.

The whitening/bleaching agents may be an enzyme, such as an oxidoreductase. Examples of suitable teeth bleaching enzymes are described in WO 97/06775 (from Novo Nordisk A/S).

Water

Water is usually added in an amount giving *e.g.* toothpaste a flowable form.

Additional agents

Further water-soluble anti-bacterial agents, such as chlorhexidine digluconate, hexetidine, alexidine, quaternary ammonium anti-bacterial compounds and water-soluble sources of certain metal ions such as zinc, copper, silver and stannous (*e.g.* zinc, copper and stannous chloride, and silver nitrate), may also be included.

Also contemplated according to the invention is the addition of compounds which can be used as fluoride source, dyes/colorants, preservatives, vitamins, pH-adjusting agents, anti-caries agents, desensitizing agents etc.

Enzymes

Other essential components used in oral care products and in oral care products of the invention are enzymes. Enzymes are biological catalysts of chemical reactions in living systems. Enzymes combine with the substrates on which they act forming an intermediate enzyme-substrate complex. This complex is then converted to a reaction product and a liberated enzyme which continue its specific enzymatic function.

Enzymes provide several benefits when used for cleansing of the oral cavity. Proteases break down salivary proteins, which are adsorbed onto the tooth surface and form the pellicle, the first layer of resulting plaque. Proteases along with lipases destroy bacteria by lysing proteins and lipids which form the

structural components of bacterial cell walls and membranes. Dextranase breaks down the organic skeletal structure produced by bacteria that forms a matrix for bacterial adhesion. Proteases and amylases, not only prevents plaque formation, but also prevents the development of calculus by breaking-up the carbohydrate-protein complex that binds calcium, preventing mineralization.

Toothpaste

A toothpaste produced from an oral care composition of the invention (in weight % of the final toothpaste composition) may typically comprise the following ingredients:

Abrasive material	10 to 70%
Humectant	0 to 80%
Thickener	0.1 to 20%
Binder	0.01 to 10%
Sweetener	0.1% to 5%
Foaming agent	0 to 15%
Whitener	0 to 5%
Enzymes	0.0001% to 20%

In a specific embodiment of the invention the oral care product is toothpaste having a pH in the range from 6.0 to about 8.0 comprising

a)	10% to 70%	Abrasive material
b)	0 to 80%	Humectant
c)	0.1 to 20%	Thickener
d)	0.01 to 10%	Binder
e)	0.1% to 5%	Sweetener
f)	0 to 15%	Foaming agent
g)	0 to 5%	Whitener
i)	0.0001% to 20%	Enzymes.

Said enzymes referred to under i) include the recombinant mutanase of the invention, and optionally other types of enzymes mentioned above known to be used in toothpastes and the like.

Mouth wash

A mouth wash produced from an oral care composition of the invention (in weight % of the final mouth wash composition) may typically comprise the following ingredients:

0-20%	Humectant
0-2%	Surfactant
0-5%	Enzymes
0-20%	Ethanol
0-2%	Other ingredients (e.g. flavour, sweetener active ingredients such as fluorides).
0-70%	Water

The mouth wash composition may be buffered with an appropriate buffer *e.g.* sodium citrate or phosphate in the pH-range 6-7.5.

The mouth wash may be in none-diluted form (*i.e.* must be diluted before use).

Method of Manufacture

The oral care composition and products of the present invention can be made using methods which are common in the oral product area.

According to the present invention the recombinant mutanase and/or the substantially purified mutanase free of active contaminants can be use in food, feed and/or pet food products.

MATERIALS AND METHODS

Materials

Micro-organisms

Trichoderma harzianum CBS 243.71

A. oryzae JaL 125: *Aspergillus oryzae* IFO 4177 available from Institute for Fermentation, Osaka; 17-25 Juso Hammachi 2-Chome Yodogawa-ku, Osaka, Japan, having the alkaline protease gene named "alp" (described by Murakami K et al., (1991), Agric. Biol. Chem. 55, p. 2807-2811) deleted by a one step gene replacement method (described by G. May in "Applied Molecular Genetics of Filamentous Fungi" (1992), p. 1-25. Eds. J. R. Kinghorn and G.

Turner; Blackie Academic and Professional), using the *A. oryzae* pyrG gene as marker.

E. coli DH5α

Plasmids and Vectors:

pMT1796 (Figure 1 and Figure 2)

pMT1802 (Figure 2)

pMT1815 (Figure 2)

pHD414: *Aspergillus* expression vector is a derivative of the plasmid p775 (described in EP 238.023). The construction of the pHD414 is further described in WO 93/11249. pHD414 contains the *A. niger* glucoamylase terminator and the *A. oryzae* TAKA amylase promoter.

pHD414+mut (Figure 3)

pHan37 containing the TAKA:TPI promoter

Linkers:

Linker #1:

```
GATCCTCACA ATG TTG GGC GTT GTC CGC CGT CTA GGC CTA GG
    GAGTGT TAC AAC CCG CAA CAG GCT GCA GAT CCG GAT CCG C
           Met Leu Gly Val Val Arg Arg Leu Gly Leu Gly
```

Linker #2:

```
          C CAA TAC TGT TAG T
     GT ACG GTT ATG ACA ATC AGATC
    Ala Cys Gln Tyr Cys ***
```

Primers:

Primer 1: 5' GGGGGGATCCACCATGAG 3' (SEQ ID No. 3)

Primer 2: 5' ACGGTCAGCAGAAGAAGCTCGACGAATAGGACTGGC 3' (SEQ ID No. 4)

Primer 3: 5' GCCAGTCCTATTCGTCGAGCTTCTTCTGCTGACCGT 3' (SEQ ID No. 5)

Primer 4: 5' CCACGGTCACCAACAATAC 3' (SEQ ID No. 6)

Primer 5: GGGGGGATCCACCATGAG (SEQ ID No. 7),

Primer 6: ACGGTCAGCAGAAGAAGCTCGACGAATAGGACTGGC (SEQ ID No. 8)

Primer 7: GCCAGTCCTATTCGTCGAGCTTCTTCTGCTGACCGT (SEQ ID NO. 9),

Primer 8: CCACGGTCACCAACAATAC (SEQ ID No. 10).

Enzymes:

lysyl-specific protease from *Achromobacter Trichoderma harzianum* CBS 243.71 fermentation broth (Batch no. PPM 3897)

Media, Substrates and Solutions:

YPM: 2% maltose, 1% bactopeptone and 0.5% yeast extract)

DAPI: 4',6-diamidino-2-phenylindole (Sigma D-9542)

Britton-Robinson Buffer

BHI: Brain Heart Infusion broth

Equipment:

10 kDa cut-off ultra-filtration cassette (Alpha Minisette from Filtron).

Phenyl-sepharose FF (high sub) column (Pharmacia)

Seitz EK1 filter plate

Q-sepharose FF column (Pharmacia)

Applied Biosystems 473A protein sequencer

2 litre Kieler fermenter

Olympus model BX50 microscope

Malthus Flexi M2060 (Malthus Instrument Limited)

Methods:

Molecular biology procedures

All molecular biology procedures including restriction digests, DNA ligations, *E. coli* transformations, DNA isolations, Southern hybridizations, PCR amplifications, and library constructions and screenings were completed using standard techniques (Sambrook, J., Fritsch, E. F., and Maniatis, T. 1989. *Molecular cloning: A laboratory manual*/E.F. Cold Spring Harbor Laboratory Press, Plainview, NY).

Preparation of Mutan

Mutan is prepared by growing *Streptococcus mutans* CBS 350.71 at pH 6.5, 37°C (kept constant), and with an aeration rate of 75 rpm in a medium comprised of the following components:

NZ-Case	6.5 g/litre
Yeast Extract	6 g/litre
$(NH_4)_2SO_4$	20 g/litre
K_2PO_4	3 g/litre
Glucose	50 g/litre
Pluronic PE6100	0.1%

After 35 hours, sucrose is added to a final concentration of 60 g/litre to induce glucosyltransferase. The total fermentation time is 75 hours. The supernatant from the fermentation is centrifuged and filtered (sterile). Sucrose is then added to the supernatant to a final concentration of 5% (pH is adjusted to pH 7.0 with acetic acid) and the solution is stirred overnight at 37°C. The solution is filtered and the insoluble mutan is harvested on propex and washed extensively with deionized water containing 1% sodium benzoate, pH 5 (adjusted with acetic acid). Finally, the insoluble mutan is lyophilized and ground.

Determination of mutanase activity (MU)

One Mutanase Unit (MU) is the amount of enzyme which under standard conditions liberates 1 µmol reducing sugar (calculated as glucose) per minute. Reducing sugars were measured with alkaline $K_3Fe(CN)_6$.

Standard Conditions

Substrate.....1.5% mutan
Reaction time.....15 minutes
Temperature.....40°C
pH.....5.5

A detailed description of Novo Nordisk's analytical method (AF 180/1-GB) is available from Novo Nordisk A/S on request.

Mutanase Plate Assay

A 5% mutan suspension is made in 50 mM sodium acetate, pH 5.5 and the suspension is homogenised for 15 minutes in an Ultra Turrax T25 homogenizer at 4°C. 1% agarose in 50 mM sodium acetate, pH 5.5 is made 0.2% with respect to mutan and 12.5 ml agarose is casted in each petri dish (d=10 cm). The sample to be

analyzed for mutanase activity is applied in sample wells punched in the agarose, and the plate is incubated overnight at 37°C, whereafter clearing zones are formed around mutanase containing samples.

Western hybridization

Western hybridizations are performed using the ECL western blotting system (Amersham International, plc, Buckinghamshire, England) and a primary antibody solution containing polyclonal rabbit-anti-mutanase. The limit of detection is 0.001 MU/ml.

Mass spectrometry

Mass spectrometry of purified wild-type mutanase is done using matrix assisted laser desorption ionization time-of-flight mass spectrometry in a VG Analytical TofSpec. For mass spectrometry 2 ml of sample is mixed with 2 ml saturated matrix solution (a-cyano-4-hydroxycinnamic acid in 0.1% TFA:acetonitrile (70:30)) and 2 ml of the mixture spotted onto the target plate. Before introduction into the mass spectrometer the solvent is removed by evaporation. Samples are desorbed and ionized by 4 ns laser pulses (337 nm) at threshold laser power and accelerated into the field-free flight tube by an accelerating voltage of 25 kV. Ions are detected by a microchannel plate set at 1850 V.

Preparation of Hydroxyapatite disks (HA)

Hydroxyapatite tablets are prepared by compressing 250 mg of hydroxyapatite in a tablet die at about 5,900 kg (13,000 lbs) of pressure for 5 minutes. The tablets are then sintered at 600°C for 4 hours and finally hydrated with sterile deionized water.

Plaque coating of Hydroxyapatite disks (HA)

Hydroxyapatite disks (HA) were dry sterilised (121°C, 2 bar, 20 minutes) and coated with filter sterilised saliva for 18 hours at 37°C. The HA disks were placed in a sterile rack in a beaker, Brain Heart Infusion broth (BHI) containing 0.2% sucrose was poured into the beaker covering the disks. Sterile Na_2S (pH 7.0) was added immediately before inoculation given the final concen-

tration of 5 g/litre. A mixture 1:1:1 of *Streptococcus mutans*, *Actinomyces viscosus* and *Fusobacterium nucleatum* grown anaerobically (BHI, 37°C, 24 h) was used as inoculum in the concentration of approximately 10^6 cfu/ml. The disks were incubated anaerobic at 37°C for 4 days with slight stirring.

Malthus-method for plaque

The Malthus-method is based on the methods described in Johnston et al., (1995), Journal of Microbiological Methods 21, p. 15-26 and Johansem et al. (1995), Journal of Applied Bacteriology 78, p. 297-303.

EXAMPLES

Example 1

Purification of wild-type Mutanase

100 g fermentation broth of *Trichoderma harzianum* CBS 243.71 (Batch no. PPM 3897) were dissolved in 1 litre 10 mM sodium acetate, pH 5.2 overnight at 4°C.

65 g DEAE-Sephadex A-50 were swelled in 3 litre 10 mM sodium acetate, pH 5.2. Excess buffer was removed after swelling. DEAE-Sephadex was mixed with the crude mutanase preparation for 1 hour and unbound material was collected by filtration through Propex cloth. The gel was further washed with 2.5 l of 10 mM sodium acetate, pH 5.2. A pool containing the unbound material was made; volume 4 litre. Remaining DEAE-Sephadex particles were removed by filtration through a Whatman GF/F filter.

350 ml S-Sepharose was equilibrated in 10 mM sodium acetate, pH 5.2 and mixed with 600 ml of the pool from the DEAE-Sephadex for 10 minutes. Unbound material was collected by filtration through Propex cloth and the gel was washed with 500 ml 10 mM sodium acetate buffer, pH 5.2. Bound material was eluted with the same buffer containing 1 M NaCl. The procedure was repeated 7 times. The combined pool containing the unbound material (7 litre) was concentrated on a Filtron concentrator equipped with a 10 kDa cut-off membrane and followed by a buffer change to 10 mM sodium acetate, pH 4.7. The concentrate was filtrated through a

Whatmann GF/F filter. The final volume of the concentrate was 600 ml.

An S-Sepharose column (180 ml, 2.6 x 33 cm) was equilibrated with 10 mM sodium acetate, pH 4.7. The pH adjusted concentrate from the S-Sepharose batch ion exchange was applied onto the column in 50 ml portions with a flow of 10 ml/min. The mutanase was eluted with a linear gradient from 0 to 20 mM NaCl in 3 column volumes. The residual protein was eluted with the same buffer containing 1 M NaCl. Fractions were analyzed for mutanase activity (plate assay) and fractions with high activity were pooled. The procedure was repeated 12 times. The combined mutanase pool was concentrated in a Filtron concentrator equipped with a 10 kDa cut-off membrane and followed by a buffer change to 10 mM Tris-HCl, pH 8.0. The final volume of the concentrate was 870 ml.

The concentrated pool from the S-Sepharose column was further purified on a HiLoad Q-Sepharose column (50 ml, 2.6 x 10 cm) equilibrated with 10 mM Tris-HCl, pH 8.0. Portions of 130 ml was applied with a flow of 8 ml/min. Elution of the mutanase was performed with a linear gradient from 0 to 50 mM NaCl in 12 column volumes. Fractions with high mutanase activity (plate assay) were pooled, concentrated in an Amicon cell equipped with a 10 kDa cut-off membrane. Finally, the mutanase preparation was dialyzed extensively against 10 mM sodium phosphate, pH 7.0 and filtrated through a 0.45 mm filter.

The yield of the mutanase in the purification described above was 300 mg. The purity of the HiLoad-Q preparation was analyzed by SDS-PAGE and N-terminal sequencing and judged by both methods the purity was around 95%.

Example 2

N-terminal sequencing of wild-type Mutanase

N-terminal amino acid sequencing was carried out in an Applied Biosystems 473A protein sequencer.

To generate peptides reduced and S-carboxymethylated mutanase (» 450 mg) was digested with the lysyl-specific protease from *Achromobacter* (10 mg) in 20 mM NH_4HCO_3 for 16 hours at 37°C. The

resulting peptides were separated by reversed phase HPLC using a Vydac C_{18} column eluted with a linear gradient of 80% 2-propanol containing 0.08% TFA in 0.1% aqueous TFA. Peptides were repurified by reversed-phase-HPLC using a Vydac C_{18} column eluted with linear gradients of 80% acetonitrile containing 0.08% TFA in 0.1% aqueous TFA before being subjected to N-terminal amino acid sequencing.

The amino acid sequences determined are given below.

N-terminal:

Ala-Ser-Ser-Ala-Asp-Arg-Leu-Val-Phe-Cys-His-Phe-Met-Ile-Gly-Ile-Val-Gly-Asp-Arg-Gly-Ser-Ser-Ala-Asp-Tyr-Asp-Asp-Asp-

Peptide 1:

Val Phe-Ile-Ser-Phe-Asp-Phe-Asn-Trp-Trp-Ser-Pro-Gly-Asn-Ala-Val-Gly-Val-Gly-Gln-Lys

Peptide 2:

Pro-Tyr-Leu-Ala-Pro-Val-Ser-Pro-Trp-Phe-Phe-Thr-His-Phe-Gly-Pro-Glu-Val-Ser-Tyr-Ser-

Peptide 3:

Trp-Val-Asn-Asp-Met-Pro-His-Asp-Gly-Phe-Leu-Asp-Leu-Ser-Lys

Example 3

Construction of the mutanase expression vectors, pMT1796, pMT1802 and pMT1815

A cDNA clone encoding mutanase was identified in a *Trichoderma harzianum* CBS 243.71 library by hybridization with a fragment of the gene amplified by PCR using primers based on the mutanase sequence shown in SEQ ID NO. 1.

DNA sequence analysis of the isolated clone, pHD414+mut, showed that it indeed encoded the mutanase gene, and that the 5' end of the construct contained a long leader sequence. To remove this leader, pHD414+mut was restricted with the enzymes *Eco*RI, *Nar*I and *Xho*I. From this digestion a 3499 nt (nucleotide) vector fragment and a 610 nt *Nar*I/*Xho*I fragment were isolated. These two fragments were then ligated with linker #1 (see above) and a 618 nt *Eco*RI/*Bam*HI fragment from pHan37 containing the TAKA:TPI promoter, giving plasmid pJW99. HD414+mut was next digested with *Xho*I and *Sph*I, and a 1790 nt

fragment encoding amino acids 35-598 of the mutanase gene was isolated.

This fragment was ligated with linker #2 (see above) and pJW99 that had been linearized with the restriction enzymes *Xba*I and *Xho*I. The resulting plasmid, pMT1802, contains the *T. harzianum* mutanase gene under the control of the TAKA:TPI promoter. Plasmid pMT1796 is identical to pMT1802 except that E36 of the mutanase protein has been changed to K36 by replacing the *Xho*I/*Kpn*I fragment of pMT1802 with a PCR amplified fragment containing the desired mutation.

This PCR fragment was created in a two step procedure as reported in Ho, et al. (1989), Gene, 77, p. 51-59, using the following primers:

Primer 1 (nt 2751 5'CAGCGTCCACATCACGAGC nt 2769) and
Primer 2 (nt 3306 5'GAAGAAGCACGTTTCTCGAGAGACCG nt 3281);
Primer 3 (nt 3281 5' CGGTCTCTGAGAAACGTGCTTCTTC nt 3306) and
Primer 4 (nt 4266 5'GCCACTTCCGTTATTAGCC nt 4248); nucleotide numbers refer to the pMT1802 plasmid (See SEQ ID No. 11).

To create pMT1815, a 127 nt DNA fragment was PCR amplified using again a two step procedure and the primers:

Primer 5: GGGGGGATCCACCATGAG;
Primer 6: ACGGTCAGCAGAAGAAGCTCGACGAATAGGACTGGC;
Primer 7: GCCAGTCCTATTCGTCGAGCTTCTTCTGCTGACCGT;
Primer 8: CCACGGTCACCAACAATAC,

and the plasmids pHan37 and pMT1802 as templates in the first round of amplification.

This fragment contains a *Bam*HI restriction enzyme site followed by the Lipolase® prepro-sequence in frame with residues 38-54 of the mutanase protein and ending with a *Bst*EII site.

The fragment was digested with the restriction enzymes *Bst*EII and *Bam*HI and inserted into pMT1802 that had been linearized with the same pair of enzymes. Changes in constructs were confirmed and the integrity of the resulting coding regions were checked by nucleotide sequencing.

Example 4

Expression of recombinant Mutanase in *Aspergillus oryzae*

The strain *A. oryzae* JaL125 was transformed using a PEG-mediated protocol (see EP 238 023) and a DNA mixture containing 0.5 µg of a plasmid encoding the gene that confers resistance to the herbicide Basta and 8.0 µg of one of the three mutanase expression plasmids. Transformants were selected on minimal plates containing 0.5% basta and 50 mM urea as a nitrogen source.

Shake flask cultures

Transformed colonies were spore purified twice on selection media and spores were harvested. A 20 ml universal container (Nunc, cat #364211) containing 10 ml YPM (2% maltose, 1% bactopeptone and 0.5% yeast extract) was inoculated with spores and grown for 5 days with shaking at 30°C. The supernatant was harvested after 5 days growth.

Construct	highest mutanase level detected	number of transformants tested
pMT1802, mutanase prepro + mutanase	<0.001	10
pMT1796, mutanase prepro + KEX2 + mutanase	3.8	4
pMT1815, Lipolase® prepro + mutanase	0.16	22

Table 1 Comparison of mutanase expression from the three different expression constructs. The limit of detection was 0.001 MU/ml

The presence of mutanase in culture supernatants was examined by western hybridizations. SDS-PAGE and protein transfers were performed using standard protocols.

Example 5

Purification of recombinant mutanase

700 ml fermentation broth was filtered and concentrated. The pH was adjusted to 4.7 (conductivity around 300 µS/cm) and the broth was loaded onto an S-Sepharose column (XK 50/22) (Pharmacia) equilibrated in 10 mM sodium acetate pH 4.7. The

mutanase was eluted in a linear NaCl gradient. The major part of the mutanase appeared in the unbound fractions. These fractions were pooled and concentrated. Then the concentrate was loaded onto a HiLoad Q-Sepharose column (Pharmacia) equilibrated in 10 mM Tris-HCl, pH 8.0 (around 600 µS/cm). The mutanase was eluted in a linear gradient of NaCl and the mutanase containing-fractions were pooled according to purity and activity. The pooled fractions were concentrated and a fraction was further purified by gelfiltration on a Superdex 75 (16/60) column (Pharmacia) in sodium acetate pH 6.0.

The purified mutanase has a specific activity around 19 MU pr. absorption unit at 280 nm. From SDS-PAGE (Novex 4-20 %; run according to the manufacturer's instructions) a molecular weight around 80 kDa is found.

The N-terminal amino acid sequence was confirmed to be identical to the N-terminal amino acid sequence of the wt mutanase (Ala-Ser-Ser-Ala-) (see Example 2)

Example 6

pH-profile of mutanase

500 ml 5 % mutan in 50 mM Britton-Robinson buffer at varying pH was added 2 ml enzyme sample (diluted in MilliQ-filtered water) in large vials (to ensure sufficient agitation) and incubated for 15 minutes at 40°C while shaking vigorously. The reaction was terminated by adding 0.5 ml 0.4 M NaOH and the samples were filtered on Munktell filters. 100 µl filtrate in Eppendorf vials were added 750 µl ferricyanide reagent (0.4 g/l $K_3Fe(CN)_6$, 20 g/l Na_2CO_3) and incubated 15 minutes at 85°C. After allowing the samples to cool, the decrease in absorption at 420 nm was measured. A dilution series of glucose was included as a standard. Substrate and enzyme blanks were always included. Samples were run in duplicate. The pH-optimum for both wild-type and recombinant enzyme is around pH 3.5-5.5 (see Figure 4).

Example 7

Temperature profile of mutanase:

500 ml 5 % mutan in 100 mM sodium acetate, pH 5.5 or in 100 mM sodium phosphate, pH 7 was added.2 ml enzyme sample (diluted in MilliQ-filtered water) in large vials (to ensure sufficient agitation) and incubated for 15 minutes at various temperatures while shaking vigorously. The reaction was terminated by adding 0.5 ml 0.4 M NaOH and the samples were filtered on Munktell filters. 100 µl filtrate in Eppendorf vials were added 750 µl ferricyanide reagent (0.4 g/l $K_3Fe(CN)_6$, 20 g/l Na_2CO_3) and incubated 15 minutes at 85°C. After allowing the samples to cool, the drop in absorption at 420 nm was measured. A dilution series of glucose was included as a standard. Substrate and enzyme blanks were always included. Samples were run in duplicate. The temperature profiles for the recombinant and wt mutanase were identical. The temperature optimum at pH 7 was around 45 °C. The temperature optimum at pH 5.5 was above 55° (See Figure 5).

Example 8

Temperature stability of mutanase:

The temperature stability was investigated by pre-incubating enzyme samples for 30 minutes at various temperatures in 0.1 M sodium acetate, pH 5.5 or in 0.1 M sodium phosphate, pH 7 before assaying the residual activity. Both recombinant and wt mutanase have similar temperature stability profiles. The residual activity starts to decline at 40 °C at pH 7, while the enzyme is more stable at pH 5.5, where the residual activity starts to decline at 55°C (See Figure 6).

Example 9

Molecular weight of purified wild-type Mutanase

The mass spectrometry, performed as described above, of the mutanase revealed an average mass around 75 kDa. In addition, it was clear from the spectra that the glycosylation of the mutanase is heterogeneous. The peptide mass of the mutanase is more than 64 kDa meaning that more than 11 kDa of carbohydrate is attached

to the enzyme.

Example 10

Activity of mutanase against Dental Plaque

A plaque biofilm was grown anaerobic on saliva coated hydroxyapatite disks as described in the Material and Methods Section above. The plaque was a mixed culture of *Streptococcus mutans* (SFAG, CBS 350.71), *Actinomyces viscosus* (DSM 43329) and *Fusobacterium nucleatum* subsp. *polymorphum* (DSM 20482).

HA disks with plaque were transferred to acetate buffer (pH 5.5) containing recombinant *Trichoderma* mutanase 1 MU/ml and whirled for 2 minutes (sterile buffer was used as control).

After enzyme treatment, the disks were either DAPI stained or transferred to Malthus cells, as indirect impedance measurements were used when enumerating living adherent cells (Malthus Flexi M2060, Malthus Instrument Limited).

For the impedance measurements 3 ml of BHI were transferred to the outer chamber of the indirect Malthus cells, and 0.5 ml of sterile KOH (0.1 M) was transferred to the inner chamber. After mutanase treatment the disks with plaque were slightly rinsed with phosphate buffer and transferred to the outer chamber. The detection times (dt) in Malthus were converted to colony counts by use of a calibration curve relating cfu/ml to dt (Figure 7).

The calibration curve was constructed by a series of 10-fold dilution rate prepared from the mixed culture. Conductance dt of each dilution step was determined in BHI and a calibration curve relating cfu/ml of the 10 fold dilutions to dt in BHI was constructed for the mixed culture (Figure 7).

The removal of plaque from the disks was also determined by fluorescent microscopy, after mutanase treatment disks were stained with DAPI (3 mM) and incubated in the dark for 5 minutes (20°C). The DAPI stained cells were examined with the x 100 oil immersion fluorescence objective on an Olympus model BX50 microscope equipped with a 200 W mercury lamp and an UV- filter. The result was compared with the quantitative data obtained by the impedance measurements.

The number of living cells on the saliva treated HA-surface after enzyme treatment was determined by the Malthus method and shown in Table 1. However, by the Malthus method it is not possible to distinguish between a bactericidal activity of mutanase or an enzymatic removal of the plaque. Therefore a decrease in living bacteria on the surface has to be compared with the simultaneously removal of plaque from the surface which is estimated by the DAPI staining.

Mutanase (MU/ml)	Log_{10} reduction (cfu/cm^2)	Removal of plaque (%)	No. of observations
0	0	0	10
1	1.4	96	6

Table 2: Enzymatic plaque removal (pH 5.5, 2 minutes) from saliva treated hydroxyapatite determined by impedance measurements.

A significant removal of plaque was determined by fluorescent microscopy after treatment with mutanase. Thus mutanase reduced the amount of adhering cells. However, the activity was observed as a removal of plaque and not as a bactericidal activity against cells in plaque.

SEQUENCE LISTING

(1) GENERAL INFORMATION:
(i) APPLICANT:
(A) NAME: Novo Nordisk A/S
(B) STREET: Novo Alle
(C) CITY: Bagsvaerd
(E) COUNTRY: Denmark
(F) POSTAL CODE (ZIP): DK-2880
(G) TELEPHONE: +45 4444 8888
(H) TELEFAX: +45 4449 3256
(ii) TITLE OF INVENTION: A recombinant enzyme with mutanase activity
(iii) NUMBER OF SEQUENCES: 11
(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)

(2) INFORMATION FOR SEQ ID NO: 1:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1905 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: DNA (genomic)
(vi) ORIGINAL SOURCE:
(B) STRAIN: Trichoderma harzianum CBS 243.71
(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION:1..1905
(A) NAME/KEY: sig_peptide
(B) LOCATION:1..120
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

```
ATG TTG GGC GTT GTC CGC CGT CTA GGC CTA GGC GCC CTT GCT GCC GCA        48
Met Leu Gly Val Val Arg Arg Leu Gly Leu Gly Ala Leu Ala Ala Ala
 1               5                  10                  15

GCT CTG TCT TCT CTC GGC AGT GCC GCT CCC GCC AAT GTT GCT ATT CGG        96
Ala Leu Ser Ser Leu Gly Ser Ala Ala Pro Ala Asn Val Ala Ile Arg
            20                  25                  30

TCT CTC GAG GAA CGT GCT TCT TCT GCT GAC CGT CTC GTA TTC TGT CAC       144
Ser Leu Glu Glu Arg Ala Ser Ser Ala Asp Arg Leu Val Phe Cys His
        35                  40                  45

TTC ATG ATT GGT ATT GTT GGT GAC CGT GGC AGC TCA GCA GAC TAT GAT       192
Phe Met Ile Gly Ile Val Gly Asp Arg Gly Ser Ser Ala Asp Tyr Asp
    50                  55                  60

GAT GAC ATG CAA CGT GCC AAA GCC GCT GGC ATT GAC GCA TTC GCT CTG       240
Asp Asp Met Gln Arg Ala Lys Ala Ala Gly Ile Asp Ala Phe Ala Leu
65                  70                  75                  80

AAC ATC GGC GTT GAC GGC TAT ACC GAC CAG CAA CTC GGG TAT GCC TAT       288
Asn Ile Gly Val Asp Gly Tyr Thr Asp Gln Gln Leu Gly Tyr Ala Tyr
                85                  90                  95

GAC TCT GCC GAC CGT AAT GGC ATG AAA GTC TTC ATT TCA TTC GAT TTC       336
Asp Ser Ala Asp Arg Asn Gly Met Lys Val Phe Ile Ser Phe Asp Phe
            100                 105                 110

AAC TGG TGG AGC CCC GGT AAT GCA GTT GGT GTT GGC CAG AAG ATT GCG       384
Asn Trp Trp Ser Pro Gly Asn Ala Val Gly Val Gly Gln Lys Ile Ala
        115                 120                 125
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CAG TAT GCC AGC CGT CCC GCC CAG CTG TAT GTT GAC AAC CGG CCA TTC          432
Gln Tyr Ala Ser Arg Pro Ala Gln Leu Tyr Val Asp Asn Arg Pro Phe
    130                 135                 140

GCC TCT TCC TTC GCT GGT GAC GGT TTG GAT GTA AAT GCG TTG CGC TCT          480
Ala Ser Ser Phe Ala Gly Asp Gly Leu Asp Val Asn Ala Leu Arg Ser
145                 150                 155                 160

GCT GCA GGC TCC AAC GTT TAC TTT GTG CCC AAC TTC CAC CCT GGT CAA          528
Ala Ala Gly Ser Asn Val Tyr Phe Val Pro Asn Phe His Pro Gly Gln
                165                 170                 175

TCT TCC CCC TCC AAC ATT GAT GGC GCC CTC AAC TGG ATG GCC TGG GAT          576
Ser Ser Pro Ser Asn Ile Asp Gly Ala Leu Asn Trp Met Ala Trp Asp
            180                 185                 190

AAT GAT GGA AAC AAC AAG GCA CCC AAG CCG GGC CAG ACT GTC ACG GTG          624
Asn Asp Gly Asn Asn Lys Ala Pro Lys Pro Gly Gln Thr Val Thr Val
        195                 200                 205

GCA GAC GGT GAC AAC GCT TAC AAG AAT TGG TTG GGT GGC AAG CCT TAC          672
Ala Asp Gly Asp Asn Ala Tyr Lys Asn Trp Leu Gly Gly Lys Pro Tyr
    210                 215                 220

CTA GCG CCT GTC TCC CCT TGG TTT TTC ACC CAT TTT GGC CCT GAA GTT          720
Leu Ala Pro Val Ser Pro Trp Phe Phe Thr His Phe Gly Pro Glu Val
225                 230                 235                 240

TCA TAT TCC AAG AAC TGG GTC TTC CCA GGT GGT CCT CTG ATC TAT AAC          768
Ser Tyr Ser Lys Asn Trp Val Phe Pro Gly Gly Pro Leu Ile Tyr Asn
                245                 250                 255

CGG TGG CAA CAG GTC TTG CAG CAG GGC TTC CCC ATG GTT GAG ATT GTT          816
Arg Trp Gln Gln Val Leu Gln Gln Gly Phe Pro Met Val Glu Ile Val
            260                 265                 270

ACC TGG AAT GAC TAC GGC GAG TCT CAC TAC GTC GGT CCT CTG AAG TCT          864
Thr Trp Asn Asp Tyr Gly Glu Ser His Tyr Val Gly Pro Leu Lys Ser
        275                 280                 285

AAG CAT TTC GAT GAT GGC AAC TCC AAA TGG GTC AAT GAT ATG CCC CAT          912
Lys His Phe Asp Asp Gly Asn Ser Lys Trp Val Asn Asp Met Pro His
    290                 295                 300

GAT GGA TTC TTG GAT CTT TCA AAG CCG TTT ATT GCT GCA TAT AAG AAC          960
Asp Gly Phe Leu Asp Leu Ser Lys Pro Phe Ile Ala Ala Tyr Lys Asn
305                 310                 315                 320

AGG GAT ACT GAT ATA TCT AAG TAT GTT CAA AAT GAG CAG CTT GTT TAC         1008
Arg Asp Thr Asp Ile Ser Lys Tyr Val Gln Asn Glu Gln Leu Val Tyr
                325                 330                 335

TGG TAC CGC CGC AAC TTG AAG GCA TTG GAC TGC GAC GCC ACC GAC ACC         1056
Trp Tyr Arg Arg Asn Leu Lys Ala Leu Asp Cys Asp Ala Thr Asp Thr
            340                 345                 350

ACC TCT AAC CGC CCG GCT AAT AAC GGA AGT GGC AAT TAC TTT ATG GGA         1104
Thr Ser Asn Arg Pro Ala Asn Asn Gly Ser Gly Asn Tyr Phe Met Gly
        355                 360                 365

CGC CCT GAT GGT TGG CAA ACT ATG GAT GAT ACC GTT TAT GTT GCC GCA         1152
Arg Pro Asp Gly Trp Gln Thr Met Asp Asp Thr Val Tyr Val Ala Ala
    370                 375                 380

CTT CTC AAG ACC GCC GGT AGC GTC ACG GTC ACG TCT GGC GGC ACC ACT         1200
Leu Leu Lys Thr Ala Gly Ser Val Thr Val Thr Ser Gly Gly Thr Thr
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385                 390                 395                 400

CAA ACG TTC CAG GCC AAC GCC GGA GCC AAC CTC TTC CAA ATC CCT GCC      1248
Gln Thr Phe Gln Ala Asn Ala Gly Ala Asn Leu Phe Gln Ile Pro Ala
                405                 410                 415

AGC ATC GGC CAG CAA AAG TTT GCT CTA ACT CGC AAC GGT CAG ACC GTC      1296
Ser Ile Gly Gln Gln Lys Phe Ala Leu Thr Arg Asn Gly Gln Thr Val
            420                 425                 430

TTT AGC GGA ACC TCA TTG ATG GAT ATC ACC AAC GTT TGC TCT TGC GGT      1344
Phe Ser Gly Thr Ser Leu Met Asp Ile Thr Asn Val Cys Ser Cys Gly
        435                 440                 445

ATC TAC AAT TTC AAC CCA TAT GTT GGC ACC ATT CCT GCC GGC TTT GAC      1392
Ile Tyr Asn Phe Asn Pro Tyr Val Gly Thr Ile Pro Ala Gly Phe Asp
    450                 455                 460

GAC CCT CTT CAG GCT GAC GGT CTT TTC TCT TTG ACC ATC GGA TTG CAT      1440
Asp Pro Leu Gln Ala Asp Gly Leu Phe Ser Leu Thr Ile Gly Leu His
465                 470                 475                 480

GTC ACG ACT TGT CAG GCC AAG CCA TCT CTT GGA ACC AAC CCT CCT GTC      1488
Val Thr Thr Cys Gln Ala Lys Pro Ser Leu Gly Thr Asn Pro Pro Val
                485                 490                 495

ACT TCT GGC CCT GTG TCC TCG CTG CCA GCT TCC TCC ACC ACC CGC GCA      1536
Thr Ser Gly Pro Val Ser Ser Leu Pro Ala Ser Ser Thr Thr Arg Ala
            500                 505                 510

TCC TCG CCT CCT GTT TCT TCA ACT CGT GTC TCT TCT CCC CCT GTC TCT      1584
Ser Ser Pro Pro Val Ser Ser Thr Arg Val Ser Ser Pro Pro Val Ser
        515                 520                 525

TCC CCT CCA GTT TCT CGC ACC TCT TCT CCC CCT CCC CCT CCG GCC AGC      1632
Ser Pro Pro Val Ser Arg Thr Ser Ser Pro Pro Pro Pro Pro Ala Ser
    530                 535                 540

AGC ACG CCG CCA TCG GGT CAG GTT TGC GTT GCC GGC ACC GTT GCT GAC      1680
Ser Thr Pro Pro Ser Gly Gln Val Cys Val Ala Gly Thr Val Ala Asp
545                 550                 555                 560

GGC GAG TCC GGC AAC TAC ATC GGC CTG TGC CAA TTC AGC TGC AAC TAC      1728
Gly Glu Ser Gly Asn Tyr Ile Gly Leu Cys Gln Phe Ser Cys Asn Tyr
                565                 570                 575

GGT TAC TGT CCA CCG GGA CCG TGT AAG TGC ACC GCC TTT GGT GCT CCC      1776
Gly Tyr Cys Pro Pro Gly Pro Cys Lys Cys Thr Ala Phe Gly Ala Pro
            580                 585                 590

ATC TCG CCA CCG GCA AGC AAT GGG CGC AAC GGC TGC CCT CTA CCG GGA      1824
Ile Ser Pro Pro Ala Ser Asn Gly Arg Asn Gly Cys Pro Leu Pro Gly
        595                 600                 605

GAA GGC GAT GGT TAT CTG GGC CTG TGC AGT TTC AGT TGT AAC CAT AAT      1872
Glu Gly Asp Gly Tyr Leu Gly Leu Cys Ser Phe Ser Cys Asn His Asn
    610                 615                 620

TAC TGC CCG CCA ACG GCA TGC CAA TAC TGT TAG                          1905
Tyr Cys Pro Pro Thr Ala Cys Gln Tyr Cys  *
625                 630                 635
```

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 635 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

Met Leu Gly Val Val Arg Arg Leu Gly Leu Gly Ala Leu Ala Ala Ala
1 5 10 15

Ala Leu Ser Ser Leu Gly Ser Ala Ala Pro Ala Asn Val Ala Ile Arg
20 25 30

Ser Leu Glu Glu Arg Ala Ser Ser Ala Asp Arg Leu Val Phe Cys His
35 40 45

Phe Met Ile Gly Ile Val Gly Asp Arg Gly Ser Ser Ala Asp Tyr Asp
50 55 60

Asp Asp Met Gln Arg Ala Lys Ala Ala Gly Ile Asp Ala Phe Ala Leu
65 70 75 80

Asn Ile Gly Val Asp Gly Tyr Thr Asp Gln Gln Leu Gly Tyr Ala Tyr
85 90 95

Asp Ser Ala Asp Arg Asn Gly Met Lys Val Phe Ile Ser Phe Asp Phe
100 105 110

Asn Trp Trp Ser Pro Gly Asn Ala Val Gly Val Gly Gln Lys Ile Ala
115 120 125

Gln Tyr Ala Ser Arg Pro Ala Gln Leu Tyr Val Asp Asn Arg Pro Phe
130 135 140

Ala Ser Ser Phe Ala Gly Asp Gly Leu Asp Val Asn Ala Leu Arg Ser
145 150 155 160

Ala Ala Gly Ser Asn Val Tyr Phe Val Pro Asn Phe His Pro Gly Gln
165 170 175

Ser Ser Pro Ser Asn Ile Asp Gly Ala Leu Asn Trp Met Ala Trp Asp
180 185 190

Asn Asp Gly Asn Asn Lys Ala Pro Lys Pro Gly Gln Thr Val Thr Val
195 200 205

Ala Asp Gly Asp Asn Ala Tyr Lys Asn Trp Leu Gly Gly Lys Pro Tyr
210 215 220

Leu Ala Pro Val Ser Pro Trp Phe Phe Thr His Phe Gly Pro Glu Val
225 230 235 240

Ser Tyr Ser Lys Asn Trp Val Phe Pro Gly Gly Pro Leu Ile Tyr Asn
245 250 255

Arg Trp Gln Gln Val Leu Gln Gln Gly Phe Pro Met Val Glu Ile Val
260 265 270

Thr Trp Asn Asp Tyr Gly Glu Ser His Tyr Val Gly Pro Leu Lys Ser
275 280 285

Lys His Phe Asp Asp Gly Asn Ser Lys Trp Val Asn Asp Met Pro His
290 295 300

Asp Gly Phe Leu Asp Leu Ser Lys Pro Phe Ile Ala Ala Tyr Lys Asn
305 310 315 320

Arg Asp Thr Asp Ile Ser Lys Tyr Val Gln Asn Glu Gln Leu Val Tyr
325 330 335

Trp Tyr Arg Arg Asn Leu Lys Ala Leu Asp Cys Asp Ala Thr Asp Thr

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            340                 345                 350

Thr Ser Asn Arg Pro Ala Asn Asn Gly Ser Gly Asn Tyr Phe Met Gly
        355                 360                 365

Arg Pro Asp Gly Trp Gln Thr Met Asp Asp Thr Val Tyr Val Ala Ala
    370                 375                 380

Leu Leu Lys Thr Ala Gly Ser Val Thr Val Thr Ser Gly Gly Thr Thr
385                 390                 395                 400

Gln Thr Phe Gln Ala Asn Ala Gly Ala Asn Leu Phe Gln Ile Pro Ala
                405                 410                 415

Ser Ile Gly Gln Gln Lys Phe Ala Leu Thr Arg Asn Gly Gln Thr Val
            420                 425                 430

Phe Ser Gly Thr Ser Leu Met Asp Ile Thr Asn Val Cys Ser Cys Gly
        435                 440                 445

Ile Tyr Asn Phe Asn Pro Tyr Val Gly Thr Ile Pro Ala Gly Phe Asp
    450                 455                 460

Asp Pro Leu Gln Ala Asp Gly Leu Phe Ser Leu Thr Ile Gly Leu His
465                 470                 475                 480

Val Thr Thr Cys Gln Ala Lys Pro Ser Leu Gly Thr Asn Pro Pro Val
                485                 490                 495

Thr Ser Gly Pro Val Ser Ser Leu Pro Ala Ser Ser Thr Thr Arg Ala
            500                 505                 510

Ser Ser Pro Pro Val Ser Ser Thr Arg Val Ser Ser Pro Pro Val Ser
        515                 520                 525

Ser Pro Pro Val Ser Arg Thr Ser Ser Pro Pro Pro Pro Pro Ala Ser
    530                 535                 540

Ser Thr Pro Pro Ser Gly Gln Val Cys Val Ala Gly Thr Val Ala Asp
545                 550                 555                 560

Gly Glu Ser Gly Asn Tyr Ile Gly Leu Cys Gln Phe Ser Cys Asn Tyr
                565                 570                 575

Gly Tyr Cys Pro Pro Gly Pro Cys Lys Cys Thr Ala Phe Gly Ala Pro

            580                 585                 590
Ile Ser Pro Pro Ala Ser Asn Gly Arg Asn Gly Cys Pro Leu Pro Gly

        595                 600                 605
Glu Gly Asp Gly Tyr Leu Gly Leu Cys Ser Phe Ser Cys Asn His Asn

    610                 615                 620

Tyr Cys Pro Pro Thr Ala Cys Gln Tyr Cys  *
625                 630                 635
```

(2) INFORMATION FOR SEQ ID NO: 3:
  (i) SEQUENCE CHARACTERISTICS:
    (A) LENGTH: 19 base pairs
    (B) TYPE: nucleic acid
    (C) STRANDEDNESS: single
    (D) TOPOLOGY: linear
  (ii) MOLECULE TYPE: other nucleic acid
    (A) DESCRIPTION: /desc = "Primer 1"

CAGCGTCCAC ATCACGAGC 19

(2) INFORMATION FOR SEQ ID NO: 4:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 26 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Primer 2"

GAAGAAGCAC GTTTCTGCAG AGACCG 26

(2) INFORMATION FOR SEQ ID NO: 5:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 26 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Primer 3"

CGGTCTCTCG AGAAACGTGC TTCTTC 26

(2) INFORMATION FOR SEQ ID NO: 6:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Primer 4"

GCCACTTCCG TTATTAGCC 19

(2) INFORMATION FOR SEQ ID NO: 7:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Primer 5"

GGGGGGATCC ACCATGAG 18

(2) INFORMATION FOR SEQ ID NO: 8:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 36 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Primer 6"

ACGGTCAGCA GAAGAAGCTC GACGAATAGG ACTGGC 36

(2) INFORMATION FOR SEQ ID NO: 9:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 36 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single

(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Primer 7"

GCCAGTCCTA TTCGTCGAGC TTCTTCTGCT GACCGT 36

(2) INFORMATION FOR SEQ ID NO: 10:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Primer 8"

CCACGGTCAC CAACAATAC 19

(2) INFORMATION FOR SEQ ID NO: 11:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 6032 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: DNA (genomic)
(vi) ORIGINAL SOURCE:
(B) STRAIN: Trichoderma harzianum CBS 243.71
(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION:3188..5092
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11:

GACGAAAGGG CCTCGTGATA CGCCTATTTT TATAGGTTAA TGTCATGATA ATAATGGTTT 60
CTTAGACGTC AGGTGGCACT TTTCGGGGAA ATGTGCGCGG AACCCCTATT TGTTTATTTT 120
TCTAAATACA TTCAAATATG TATCCGCTCA TGAGACAATA ACCCTGATAA ATGCTTCAAT 180
AATATTGAAA AAGGAAGAGT ATGAGTATTC AACATTTCCG TGTCGCCCTT ATTCCCTTTT 240
TTGCGGCATT TTGCCTTCCT GTTTTTGCTC ACCCAGAAAC GCTGGTGAAA GTAAAAGATG 300
CTGAAGATCA GTTGGGTGCA CGAGTGGGTT ACATCGAACT GGATCTCAAC AGCGGTAAGA 360
TCCTTGAGAG TTTTCGCCCC GAAGAACGTT TTCCAATGAT GAGCACTTTT AAAGTTCTGC 420
TATGTGGCGC GGTATTATCC CGTATTGACG CCGGGCAAGA GCAACTCGGT CGCCGCATAC 480
ACTATTCTCA GAATGACTTG GTTGAGTACT CACCAGTCAC AGAAAAGCAT CTTACGGATG 540
GCATGACAGT AAGAGAATTA TGCAGTGCTG CCATAACCAT GAGTGATAAC ACTGCGGCCA 600
ACTTACTTCT GACAACGATC GGAGGACCGA AGGAGCTAAC CGCTTTTTTG CACAACATGG 660
GGGATCATGT AACTCGCCTT GATCGTTGGG AACCGGAGCT GAATGAAGCC ATACCAAACG 720
ACGAGCGTGA CACCACGATG CCTGTAGCAA TGGCAACAAC GTTGCGCAAA CTATTAACTG 780
GCGAACTACT TACTCTAGCT TCCCGGCAAC AATTAATAGA CTGGATGGAG GCGGATAAAG 840
TTGCAGGACC ACTTCTGCGC TCGGCCCTTC CGGCTGGCTG GTTTATTGCT GATAAATCTG 900
GAGCCGGTGA GCGTGGGTCT CGCGGTATCA TTGCAGCACT GGGGCCAGAT GGTAAGCCCT 960
CCCGTATCGT AGTTATCTAC ACGACGGGGA GTCAGGCAAC TATGGATGAA CGAAATAGAC 1020
AGATCGCTGA GATAGGTGCC TCACTGATTA AGCATTGGTA ACTGTCAGAC CAAGTTTACT 1080
CATATATACT TTAGATTGAT TTAAAACTTC ATTTTTAATT TAAAAGGATC TAGGTGAAGA 1140
TCCTTTTTGA TAATCTCATG ACCAAAATCC CTTAACGTGA GTTTTCGTTC CACTGAGCGT 1200
CAGACCCCGT AGAAAAGATC AAAGGATCTT CTTGAGATCC TTTTTTTCTG CGCGTAATCT 1260
GCTGCTTGCA AACAAAAAAA CCACCGCTAC CAGCGGTGGT TTGTTTGCCG GATCAAGAGC 1320
TACCAACTCT TTTTCCGAAG GTAACTGGCT TCAGCAGAGC GCAGATACCA AATACTGTCC 1380
TTCTAGTGTA GCCGTAGTTA GGCCACCACT TCAAGAACTC TGTAGCACCG CCTACATACC 1440
TCGCTCTGCT AATCCTGTTA CCAGTGGCTG CTGCCAGTGG CGATAAGTCG TGTCTTACCG 1500
GGTTGGACTC AAGACGATAG TTACCGGATA AGGCGCAGCG GTCGGGCTGA ACGGGGGGTT 1560
CGTGCACACA GCCCAGCTTG GAGCGAACGA CCTACACCGA ACTGAGATAC CTACAGCGTG 1620
AGCATTGAGA AAGCGCCACG CTTCCCGAAG GGAGAAAGGC GGACAGGTAT CCGGTAAGCG 1680

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GCAGGGTCGG AACAGGAGAG CGCACGAGGG AGCTTCCAGG GGGAAACGCC TGGTATCTTT   1740
ATAGTCCTGT CGGGTTTCGC CACCTCTGAC TTGAGCGTCG ATTTTTGTGA TGCTCGTCAG   1800
GGGGGCGGAG CCTATGGAAA AACGCCAGCA ACGCGGCCTT TTTACGGTTC CTGGCCTTTT   1860
GCTGGCCTTT TGCTCACATG TTCTTTCCTG CGTTATCCCC TGATTCTGTG GATAACCGTA   1920
TTACCGCCTT TGAGTGAGCT GATACCGCTC GCCGCAGCCG AACGACCGAG CGCAGCGAGT   1980
CAGTGAGCGA GGAAGCGGAA GAGCGCCCAA TACGCAAACC GCCTCTCCCC GCGCGTTGGC   2040
CGATTCATTA ATGCAGCCTG ATTAATGATT ACATACGCCT CCGGGTAGTA GACCGAGCAG   2100
CCGAGCCAGT TCAGCGCCTA AAACGCCTTA TACAATTAAG CAGTTAAAGA AGTTAGAATC   2160
TACGCTTAAA AAGCTACTTA AAAATCGATC TCGCAGTCCC GATTCGCCTA TCAAAACCAG   2220
TTTAAATCAA CTGATTAAAG GTGCCGAACG AGCTATAAAT GATATAACAA TATTAAAGCA   2280
TTAATTAGAG CAATATCAGG CCGCGCACGA AAGGCAACTT AAAAAGCGAA AGCGCTCTAC   2340
TAAACAGATT ACTTTTGAAA AAGGCACATC AGTATTTAAA GCCCGAATCC TTATTAAGCG   2400
CCGAAATCAG GCAGATAAAG CCATACAGGC AGATAGACCT CTACCTATTA AATCGGCTTC   2460
TAGGCGCGCT CCATCTAAAT GTTCTGGCTG TGGTGTACAG GGGCATAAAA TTACGCACTA   2520
CCCGAATCGA TAGAACTACT CATTTTTATA TAGAAGTCAG AATTCATAGT GTTTTGATCA   2580
TTTTAAATTT TTATATGGCG GGTGGTGGGC AACTCGCTTG CGCGGGCAAC TCGCTTACCG   2640
ATTACGTTAG GGCTGATATT TACGTGAAAA TCGTCAAGGG ATGCAAGACC AAAGTAGTAA   2700
AACCCCGGAA GTCAACAGCA TCCAAGCCCA AGTCCTTCAC GGAGAAACCC CAGCGTCCAC   2760
ATCACGAGCG AAGGACCACC TCTAGGCATC GGACGCACCA TCCAATTAGA AGCAGCAAAG   2820
CGAAACAGCC CAAGAAAAAG GTCGGCCCGT CGGCCTTTTC TGCAACGCTG ATCACGGGCA   2880
GCGATCCAAC CAACACCCTC CAGAGTGACT AGGGGCGGAA ATTTAAAGGG ATTAATTTCC   2940
ACTCAACCAC AAATCACAGT CGTCCCCGGT ATTGTCCTGC AGAATGCAAT TTAAACTCTT   3000
CTGCGAATCG CTTGGATTCC CCGCCCCTAG TCGTAGAGCT TAAAGTATGT CCCTTGTCGA   3060
TGCGATGTAT CACAACATAT AAATACTAGC AAGGGATGCC ATGCTTGGAG TTTCCAACTC   3120
AATTTACCTC TATCCACACT TCTCTTCCTT CCTCAATCCT CTATATACAC AACTGGGGAT   3180
CCTCACA ATG TTG GGC GTT GTC CGC CGT CTA GGC CTA GGC GCC CTT GCT      3229
        Met Leu Gly Val Val Arg Arg Leu Gly Leu Gly Ala Leu Ala
          1               5                  10
GCC GCA GCT CTG TCT TCT CTC GGC AGT GCC GCT CCC GCC AAT GTT GCT      3277
Ala Ala Ala Leu Ser Ser Leu Gly Ser Ala Ala Pro Ala Asn Val Ala
 15                  20                  25                  30
ATT CGG TCT CTC GAG GAA CGT GCT TCT TCT GCT GAC CGT CTC GTA TTC      3325
Ile Arg Ser Leu Glu Glu Arg Ala Ser Ser Ala Asp Arg Leu Val Phe
             35                  40                  45
TGT CAC TTC ATG ATT GGT ATT GTT GGT GAC CGT GGC AGC TCA GCA GAC      3373
Cys His Phe Met Ile Gly Ile Val Gly Asp Arg Gly Ser Ser Ala Asp
         50                  55                  60
TAT GAT GAT GAC ATG CAA CGT GCC AAA GCC GCT GGC ATT GAC GCA TTC      3421
Tyr Asp Asp Asp Met Gln Arg Ala Lys Ala Ala Gly Ile Asp Ala Phe
     65                  70                  75
GCT CTG AAC ATC GGC GTT GAC GGC TAT ACC GAC CAG CAA CTC GGG TAT      3469
Ala Leu Asn Ile Gly Val Asp Gly Tyr Thr Asp Gln Gln Leu Gly Tyr
 80                  85                  90
GCC TAT GAC TCT GCC GAC CGT AAT GGC ATG AAA GTC TTC ATT TCA TTC      3517
Ala Tyr Asp Ser Ala Asp Arg Asn Gly Met Lys Val Phe Ile Ser Phe
 95                 100                 105                 110
GAT TTC AAC TGG TGG AGC CCC GGT AAT GCA GTT GGT GTT GGC CAG AAG      3565
Asp Phe Asn Trp Trp Ser Pro Gly Asn Ala Val Gly Val Gly Gln Lys
                115                 120                 125
ATT GCG CAG TAT GCC AGC CGT CCC GCC CAG CTG TAT GTT GAC AAC CGG      3613
Ile Ala Gln Tyr Ala Ser Arg Pro Ala Gln Leu Tyr Val Asp Asn Arg
            130                 135                 140
CCA TTC GCC TCT TCC TTC GCT GGT GAC GGT TTG GAT GTA AAT GCG TTG      3661
Pro Phe Ala Ser Ser Phe Ala Gly Asp Gly Leu Asp Val Asn Ala Leu
        145                 150                 155
CGC TCT GCT GCA GGC TCC AAC GTT TAC TTT GTG CCC AAC TTC CAC CCT      3709
Arg Ser Ala Ala Gly Ser Asn Val Tyr Phe Val Pro Asn Phe His Pro
    160                 165                 170
GGT CAA TCT TCC CCC TCC AAC ATT GAT GGC GCC CTC AAC TGG ATG GCC      3757
Gly Gln Ser Ser Pro Ser Asn Ile Asp Gly Ala Leu Asn Trp Met Ala
175                 180                 185                 190
TGG GAT AAT GAT GGA AAC AAC AAG GCA CCC AAG CCG GGC CAG ACT GTC      3805
Trp Asp Asn Asp Gly Asn Asn Lys Ala Pro Lys Pro Gly Gln Thr Val
                195                 200                 205
ACG GTG GCA GAC GGT GAC AAC GCT TAC AAG AAT TGG TTG GGT GGC AAG      3853
Thr Val Ala Asp Gly Asp Asn Ala Tyr Lys Asn Trp Leu Gly Gly Lys
            210                 215                 220
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CCT TAC CTA GCG CCT GTC TCC CCT TGG TTT TTC ACC CAT TTT GGC CCT      3901
Pro Tyr Leu Ala Pro Val Ser Pro Trp Phe Phe Thr His Phe Gly Pro
        225                 230                 235
GAA GTT TCA TAT TCC AAG AAC TGG GTC TTC CCA GGT GGT CCT CTG ATC      3949
Glu Val Ser Tyr Ser Lys Asn Trp Val Phe Pro Gly Gly Pro Leu Ile
    240                 245                 250
TAT AAC CGG TGG CAA CAG GTC TTG CAG CAG GGC TTC CCC ATG GTT GAG      3997
Tyr Asn Arg Trp Gln Gln Val Leu Gln Gln Gly Phe Pro Met Val Glu
255                 260                 265                 270
ATT GTT ACC TGG AAT GAC TAC GGC GAG TCT CAC TAC GTC GGT CCT CTG      4045
Ile Val Thr Trp Asn Asp Tyr Gly Glu Ser His Tyr Val Gly Pro Leu
                275                 280                 285
AAG TCT AAG CAT TTC GAT GAT GGC AAC TCC AAA TGG GTC AAT GAT ATG      4093
Lys Ser Lys His Phe Asp Asp Gly Asn Ser Lys Trp Val Asn Asp Met
            290                 295                 300
CCC CAT GAT GGA TTC TTG GAT CTT TCA AAG CCG TTT ATT GCT GCA TAT      4141
Pro His Asp Gly Phe Leu Asp Leu Ser Lys Pro Phe Ile Ala Ala Tyr
        305                 310                 315
AAG AAC AGG GAT ACT GAT ATA TCT AAG TAT GTT CAA AAT GAG CAG CTT      4189
Lys Asn Arg Asp Thr Asp Ile Ser Lys Tyr Val Gln Asn Glu Gln Leu
    320                 325                 330
GTT TAC TGG TAC CGC CGC AAC TTG AAG GCA TTG GAC TGC GAC GCC ACC      4237
Val Tyr Trp Tyr Arg Arg Asn Leu Lys Ala Leu Asp Cys Asp Ala Thr
335                 340                 345                 350
GAC ACC ACC TCT AAC CGC CCG GCT AAT AAC GGA AGT GGC AAT TAC TTT      4285
Asp Thr Thr Ser Asn Arg Pro Ala Asn Asn Gly Ser Gly Asn Tyr Phe
                355                 360                 365
ATG GGA CGC CCT GAT GGT TGG CAA ACT ATG GAT GAT ACC GTT TAT GTT      4333
Met Gly Arg Pro Asp Gly Trp Gln Thr Met Asp Asp Thr Val Tyr Val
            370                 375                 380
GCC GCA CTT CTC AAG ACC GCC GGT AGC GTC ACG GTC ACG TCT GGC GGC      4381
Ala Ala Leu Leu Lys Thr Ala Gly Ser Val Thr Val Thr Ser Gly Gly
        385                 390                 395
ACC ACT CAA ACG TTC CAG GCC AAC GCC GGA GCC AAC CTC TTC CAA ATC      4429
Thr Thr Gln Thr Phe Gln Ala Asn Ala Gly Ala Asn Leu Phe Gln Ile
    400                 405                 410
CCT GCC AGC ATC GGC CAG CAA AAG TTT GCT CTA ACT CGC AAC GGT CAG      4477
Pro Ala Ser Ile Gly Gln Gln Lys Phe Ala Leu Thr Arg Asn Gly Gln
415                 420                 425                 430
ACC GTC TTT AGC GGA ACC TCA TTG ATG GAT ATC ACC AAC GTT TGC TCT      4525
Thr Val Phe Ser Gly Thr Ser Leu Met Asp Ile Thr Asn Val Cys Ser
                435                 440                 445
TGC GGT ATC TAC AAT TTC AAC CCA TAT GTT GGC ACC ATT CCT GCC GGC      4573
Cys Gly Ile Tyr Asn Phe Asn Pro Tyr Val Gly Thr Ile Pro Ala Gly
            450                 455                 460
TTT GAC GAC CCT CTT CAG GCT GAC GGT CTT TTC TCT TTG ACC ATC GGA      4621
Phe Asp Asp Pro Leu Gln Ala Asp Gly Leu Phe Ser Leu Thr Ile Gly
        465                 470                 475
TTG CAT GTC ACG ACT TGT CAG GCC AAG CCA TCT CTT GGA ACC AAC CCT      4669
Leu His Val Thr Thr Cys Gln Ala Lys Pro Ser Leu Gly Thr Asn Pro
    480                 485                 490
CCT GTC ACT TCT GGC CCT GTG TCC TCG CTG CCA GCT TCC TCC ACC ACC      4717
Pro Val Thr Ser Gly Pro Val Ser Ser Leu Pro Ala Ser Ser Thr Thr
495                 500                 505                 510
CGC GCA TCC TCG CCT CCT GTT TCT TCA ACT CGT GTC TCT TCT CCC CCT      4765
Arg Ala Ser Ser Pro Pro Val Ser Ser Thr Arg Val Ser Ser Pro Pro
                515                 520                 525
GTC TCT TCC CCT CCA GTT TCT CGC ACC TCT TCT CCC CCT CCC CCT CCG      4813
Val Ser Ser Pro Pro Val Ser Arg Thr Ser Ser Pro Pro Pro Pro Pro
            530                 535                 540
GCC AGC AGC ACG CCG CCA TCG GGT CAG GTT TGC GTT GCC GGC ACC GTT      4861
Ala Ser Ser Thr Pro Pro Ser Gly Gln Val Cys Val Ala Gly Thr Val
        545                 550                 555
GCT GAC GGC GAG TCC GGC AAC TAC ATC GGC CTG TGC CAA TTC AGC TGC      4909
Ala Asp Gly Glu Ser Gly Asn Tyr Ile Gly Leu Cys Gln Phe Ser Cys
    560                 565                 570
AAC TAC GGT TAC TGT CCA CCG GGA CCG TGT AAG TGC ACC GCC TTT GGT      4957
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Asn Tyr Gly Tyr Cys Pro Pro Gly Pro Cys Lys Cys Thr Ala Phe Gly
575                 580                 585                 590
GCT CCC ATC TCG CCA CCG GCA AGC AAT GGG CGC AAC GGC TGC CCT CTA      5005
Ala Pro Ile Ser Pro Pro Ala Ser Asn Gly Arg Asn Gly Cys Pro Leu
                595                 600                 605
CCG GGA GAA GGC GAT GGT TAT CTG GGC CTG TGC AGT TTC AGT TGT AAC      5053
Pro Gly Glu Gly Asp Gly Tyr Leu Gly Leu Cys Ser Phe Ser Cys Asn
            610                 615                 620
CAT AAT TAC TGC CCG CCA ACG GCA TGC CAA TAC TGT TAG TCTAGAGGGT       5102
His Asn Tyr Cys Pro Pro Thr Ala Cys Gln Tyr Cys  *
        625                 630                 635
GACTGACACC TGGCGGTAGA CAATCAATCC ATTTCGCTAT AGTTAAAGGA TGGGGATGAG    5162
GGCAATTGGT TATATGATCA TGTATGTAGT GGGTGTGCAT AATAGTAGTG AAATGGAAGC    5222
CAAGTCATGT GATTGTAATC GACCGACGGA ATTGAGGATA TCCGGAAATA CAGACACCGT    5282
GAAAGCCATG GTCTTTCCTT CGTGTAGAAG ACCAGACAGA CAGTCCCTGA TTTACCCTGC    5342
ACAAAGCACT AGAAAATTAG CATTCCATCC TTCTCTGCTT GCTCTGCTGA TATCACTGTC    5402
ATTCAATGCA TAGCCATGAG CTCATCTTAG ATCCAAGCAC GTAATTCCAT AGCCGAGGTC    5462
CACAGTGGAG CAGCAACATT CCCCATCATT GCTTTCCCCA GGGGCCTCCC AACGACTAAA    5522
TCAAGAGTAT ATCTCTACCG TCCAATAGAT CGTCTTCGCT TCAAAATCTT TGACAATTCC    5582
AAGAGGGTCC CCATCCATCA AACCCAGTTC AATAATAGCC GAGATGCATG GTGGAGTCAA    5642
TTAGGCAGTA TTGCTGGAAT GTCGGGGCCA GTTGGCCGGG TGGTCATTGG CCGCCTGTGA    5702
TGCCATCTGC CACTAAATCC GATCATTGAT CCACCGCCCA CGAGGGCGTC TTTGCTTTTT    5762
GCGCGGCGTC CAGGTTCAAC TCTCTCCTCT AGCGCCTGAT GCGGTATTTT CTCCTTACGC    5822
ATCTGTGCGG TATTTCACAC CGCATATGGT GCACTCTCAG TACAATCTGC TCTGATGCCG    5882
CATAGTTAAG CCAGCCCCGA CACCCGCCAA CACCCGCTGA CGCGCCCTGA CGGGCTTGTC    5942
TGCTCCCGGC ATCCGCTTAC AGACAAGCTG TGACCGTCTC CGGGAGCTGC ATGTGTCAGA    6002
GGTTTTCACC GTCATCACCG AAACGCGCGA                                     6032
```

PATENT CLAIMS

1. A method for constructing an expression vector comprising a mutanase gene obtained from a filamentous fungus suitable for heterologous production comprising the steps of:

a) isolating a DNA sequence encoding a mutanase from a filamentous fungus,

b) introducing a kex2 site or kex2-like site between the DNA sequences encoding the pro-peptide and the mature region of the mutanase, or replacing the mutanase (pre)pro-sequence with a (pre)pro-sequence comprising a kex2 or kex2-like site of another fungal enzyme,

c) cloning the DNA sequence obtained in step b) into a suitable expression vector.

2. The method according to claim 1, wherein the mutanase is obtained from the genus *Trichoderma*, preferably a strain of the species *T. harzianum*, especially the strain *T. harzianum* CBS 243.71.

3. The method according to claim 2, in which the mutanase DNA sequence is isolated from or produced on the basis of a nucleic acid library of *Trichoderma harzianum* CBS 243.71.

4. The method according to any of claims 1 to 3, wherein the mutanase (pre)pro-sequence is replaced by the Lipolase® (pre)pro-sequence or the TAKA-amylase (pre)pro-sequence.

5. An expression vector comprising a mutanase gene and a DNA sequence encoding a pro-peptide with a kex2 site or kex2-like site between the DNA sequences encoding said pro-peptide and the mature region of the mutanase.

6. The expression vector according to claim 5, further comprising an operably linked promoter sequence and/or a prepro-sequence.

7. The expression vector according to claims 5 and 6, wherein the prepro-sequence comprise the original mutanase signal sequence, or the Lipolase® signal-sequence, or the TAKA pro-sequence and the original mutanase pro-sequence with a kex2 or kx2-like site, or the Lipolase® pro-sequence, or the TAKA pro-sequence.

8. The expression vector according to claim 7, wherein the promoter is the TAKA promoter or TAKA:TPI promoter.

9. The expression vector according to any claims 5 to 8, being the vector pMT1796.

10. A filamentous host cell for production of recombinant mutanase derived from a filamentous fungus being from the genus *Trichoderma*, such as a strain of *T. harzianum*, or the genus *Aspergillus*, such as a strain of *A. oryzae* or *A. niger*, or a strain of the genus Fusarium, such as a strain of *Fusarium oxysporium*, *Fusarium graminearum*, *Fusarium sulphureum*, *Fusarium cerealis*.

11. The host cell according to claim 10 wherein the host cell is a protease deficient of protease minus strain.

12. The host cell according to claim 11, wherein the host cell is the protease deficient strain *Aspergillus oryzae* JaL125 having the alkaline protease gene named "alp" deleted.

13. A process for producing a recombinant mutanase in a host cell, comprising the steps:

a) transforming an expression vector comprising a mutanase gene with a kex2 site or kex2-like site between the DNA sequences encoding the pro-peptide and the mature region of the mutanase into a suitable filamentous fungus host cell,

b) cultivating the host cell in a suitable culture medium under conditions permitting expression and secretion of an active mutanase,

c) recovering and optionally purifying the secreted active recombinant mutanase from the culture medium.

14. The process according to claim 13 wherein the recombinant expression vector is prepared according to the method of claim 1 to 4.

15. The process according to claim 13 and 14, wherein the filamentous host is a host cell according to any of claims 7 to 9.

16. An isolated recombinant mutanase produced according to the process according to any of claims 13 to 15.

17. A substantially pure wild-type mutanase obtained from *Trichoderma harzianum* CBS 243.71 essentially free of any contaminants.

18. A composition comprising a recombinant mutanase according to claim 16 or a substantially pure wild-type mutanase according to claim 17 and further other ingredients conventionally used in food, feed and/or pet food products.

19. An oral care composition comprising a recombinant mutanase according to claim 16 or a substantially pure wild-type mutanase according to claim 17, further comprising an enzyme selected from the group of dextranases, oxidases, peroxidases, haloperoxidases, laccases, proteases, endoglucosidases, lipases, amylases, and mixtures thereof.

20. An oral care product comprising a recombinant mutanase according to claim 16 or a substantially purified mutanase according to claim 17 or an oral care composition according to claim 19 and further comprising ingredients conventionally used in oral care products.

21. The oral care product according to claim 20, being a dentifrice, such as a toothpaste, tooth powder or a mouth wash.

22. Use of the recombinant mutanase according to claim 16 or the substantially purified mutanase according to claim 17 or an oral care composition of claim 19 or oral care product according to claims 20 and 21 for preventing the formation of dental plaque or removing dental plaque.

23. The use of the recombinant mutanase according to claims 16 or the substantially purified mutanase according to claim 17 or a oral care composition of claim 19 or oral care product according to claims 18 and 20 in oral care products for humans and/or animals.

24. Use of the composition according to claim 18, in food, feed and/or pet food products.

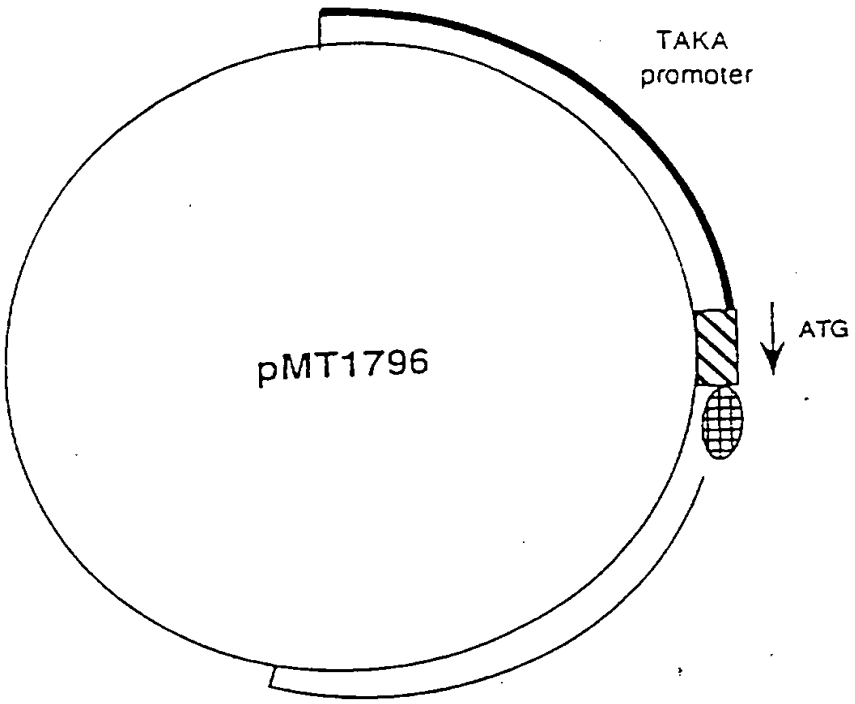

Fig. 1

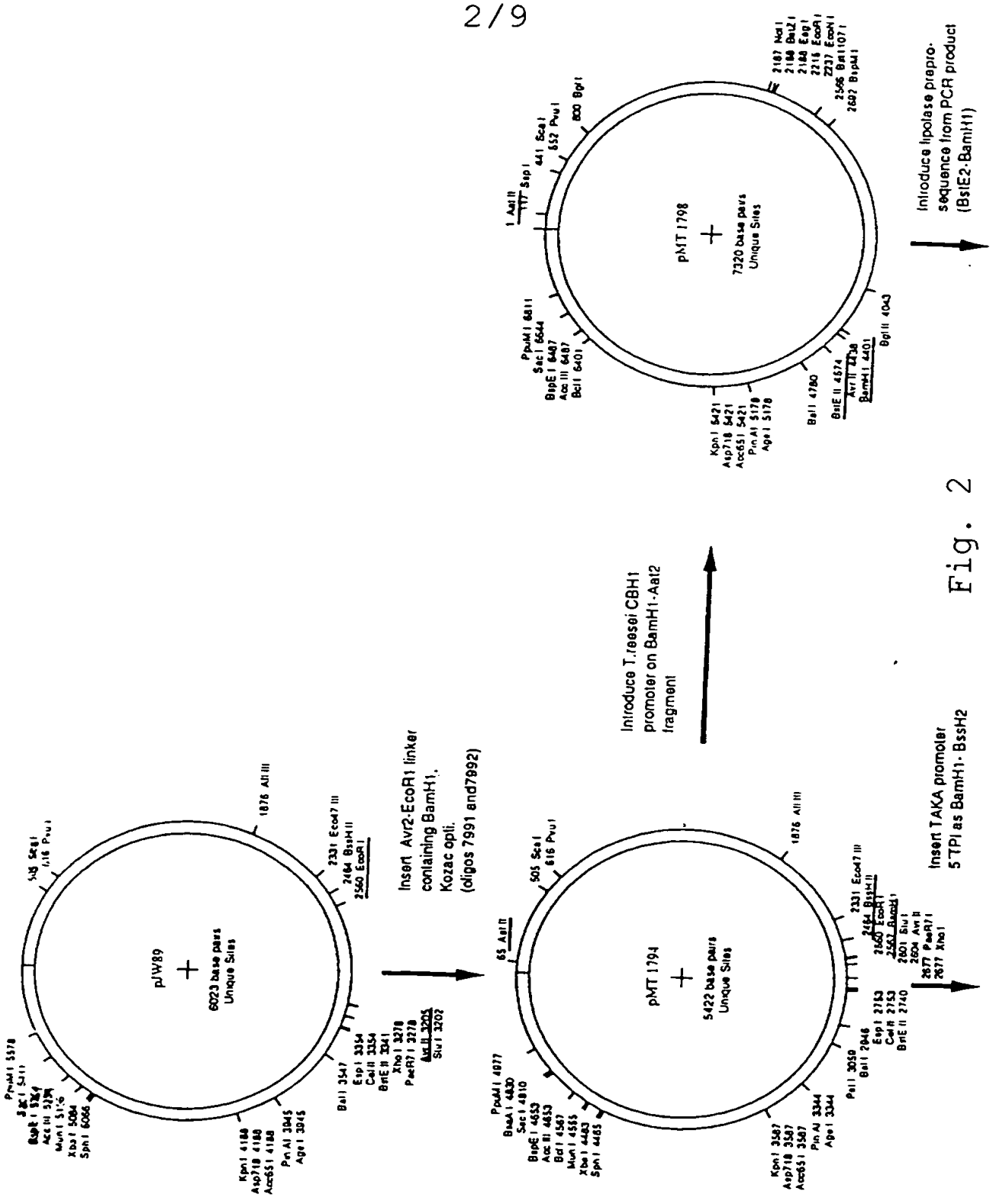

Fig. 2

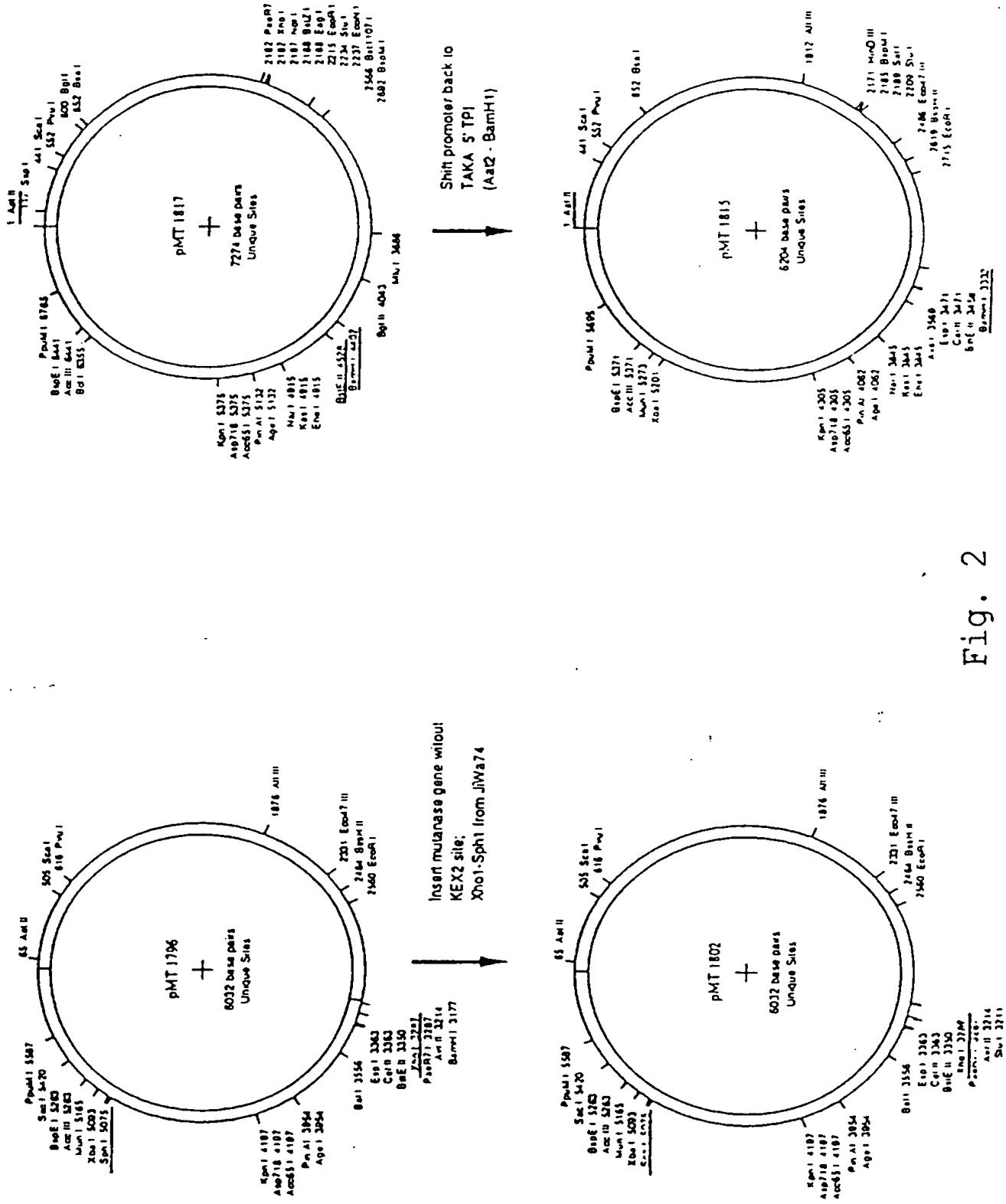

Fig. 2

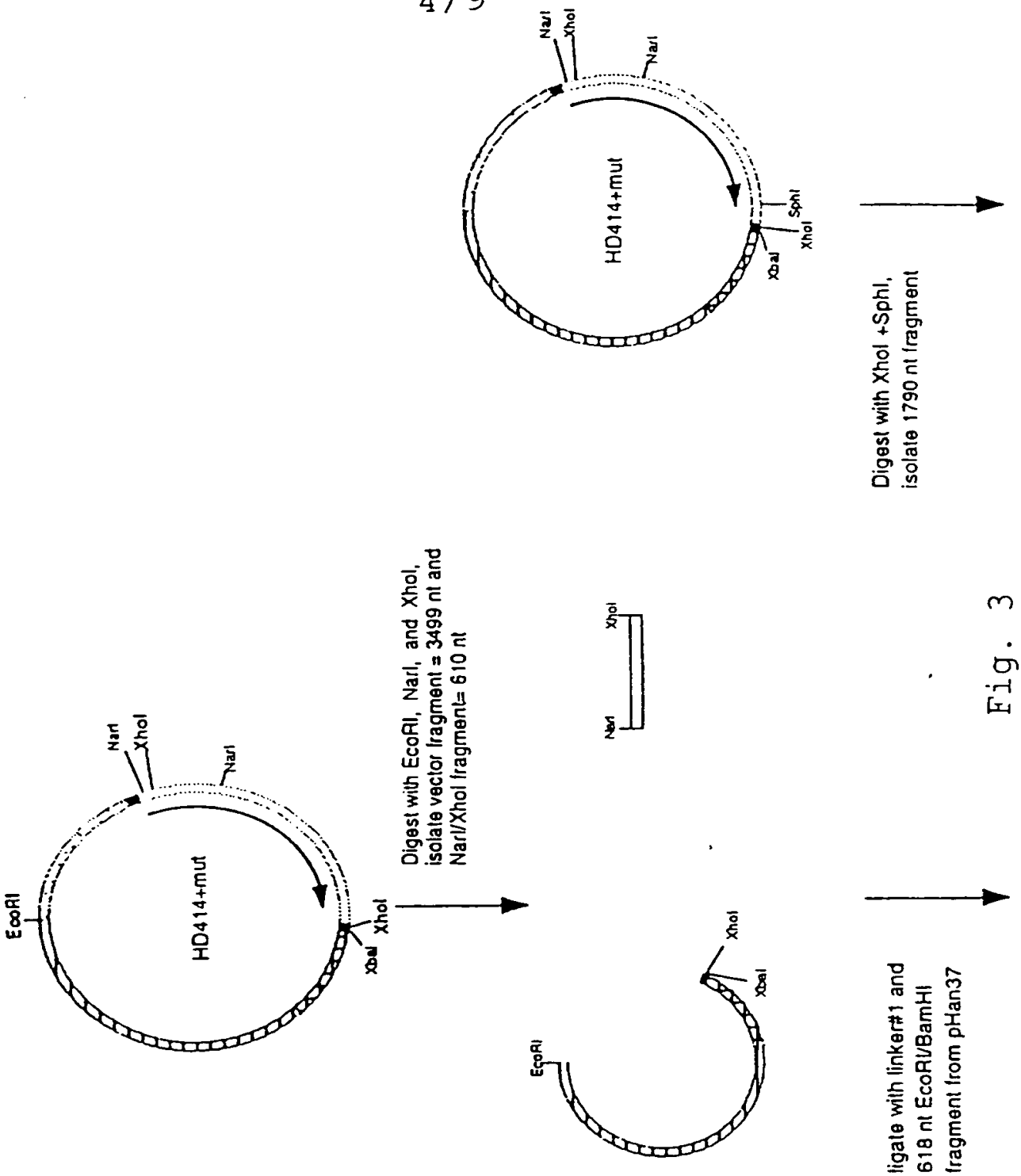
NarI
XhoI
NarI
HD414+mut
SphI
XhoI
XbaI
Digest with XhoI +SphI,
isolate 1790 nt fragment
NarI
XhoI
NarI
HD414+mut
EcoRI
XhoI
XbaI
Digest with EcoRI, NarI, and XhoI,
isolate vector fragment = 3499 nt and
NarI/XhoI fragment= 610 nt
XhoI
NarI
XhoI
XbaI
EcoRI
ligate with linker#1 and
618 nt EcoRI/BamHI
fragment from pHan37

Fig. 3

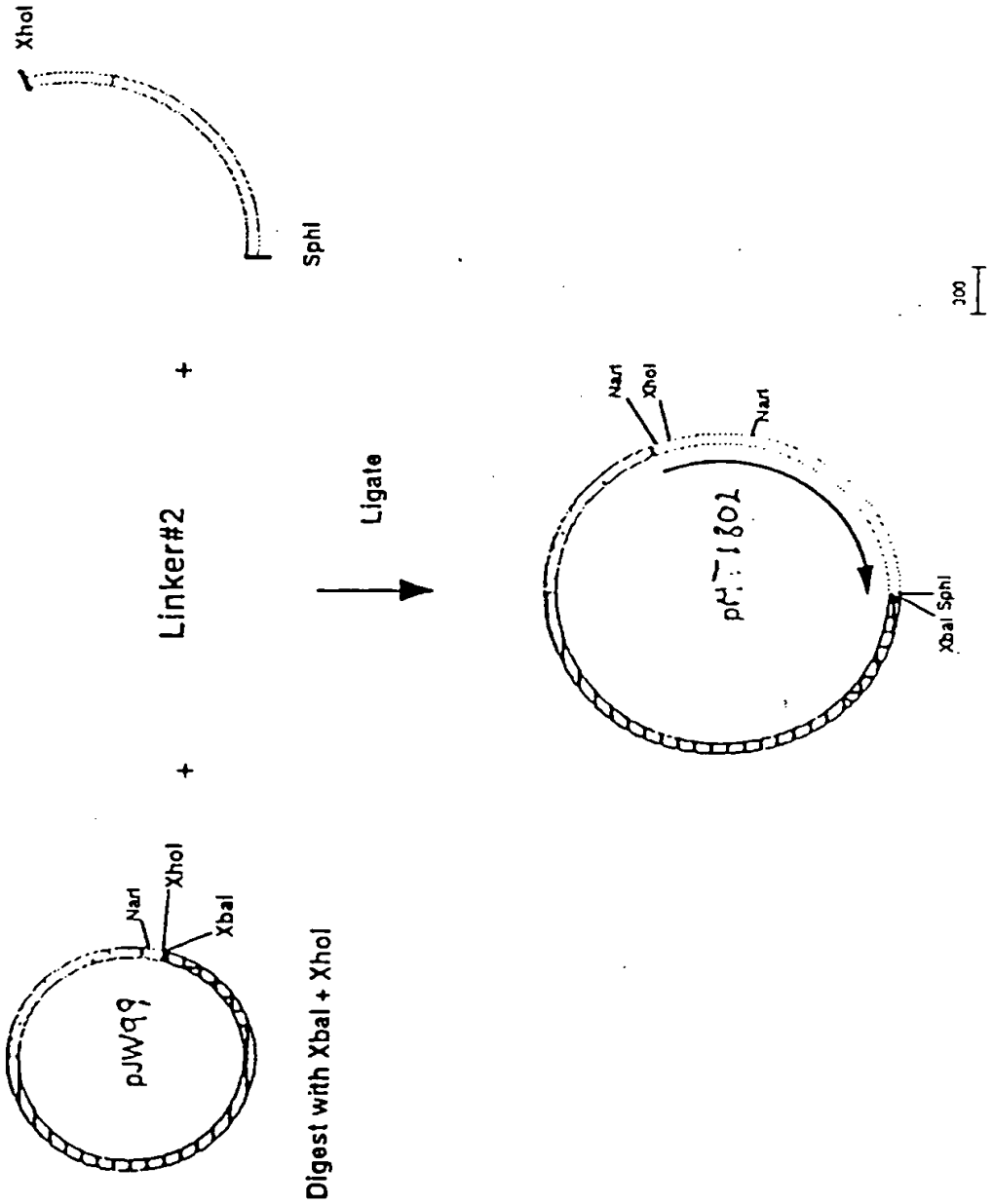

Fig. 3

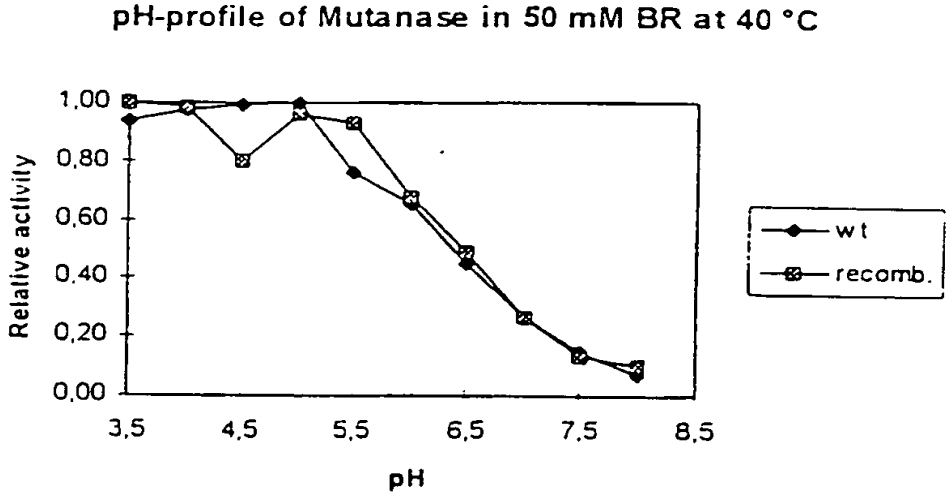

Fig. 4

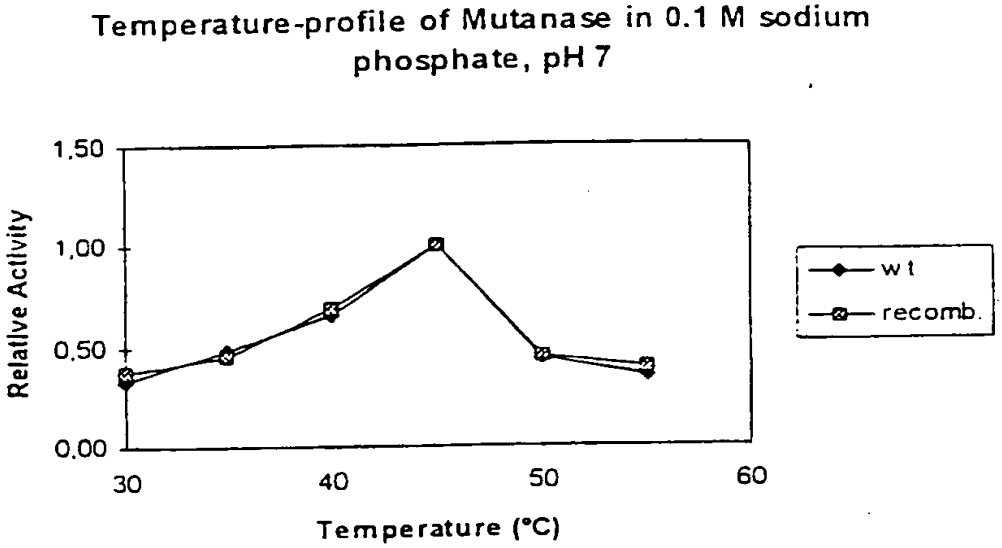

Fig. 5

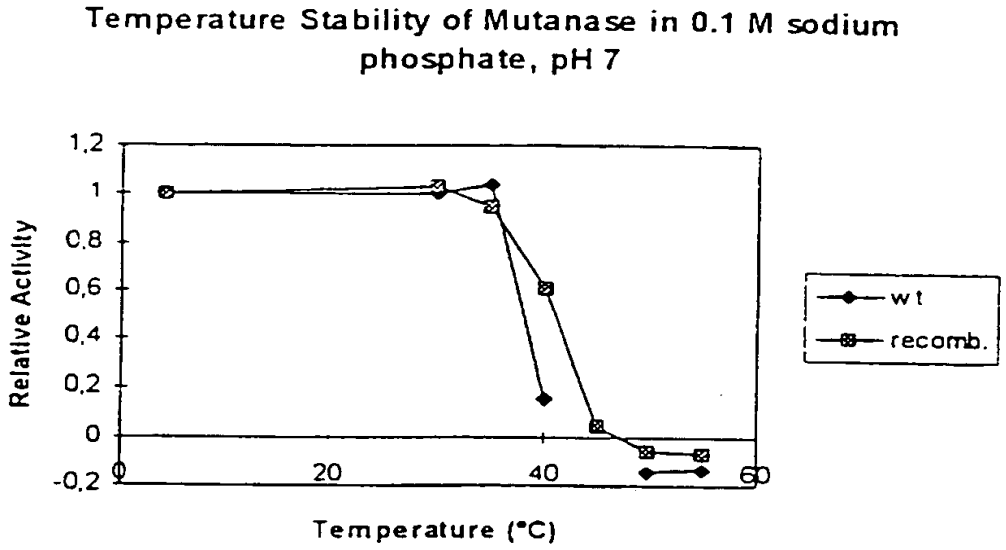
Temperature Stability of Mutanase in 0.1 M sodium phosphate, pH 7
Relative Activity
1,2
1
0,8
0,6
0,4
0,2
0
-0,2
0
20
40
60
Temperature (°C)
wt
recomb.

Fig. 6

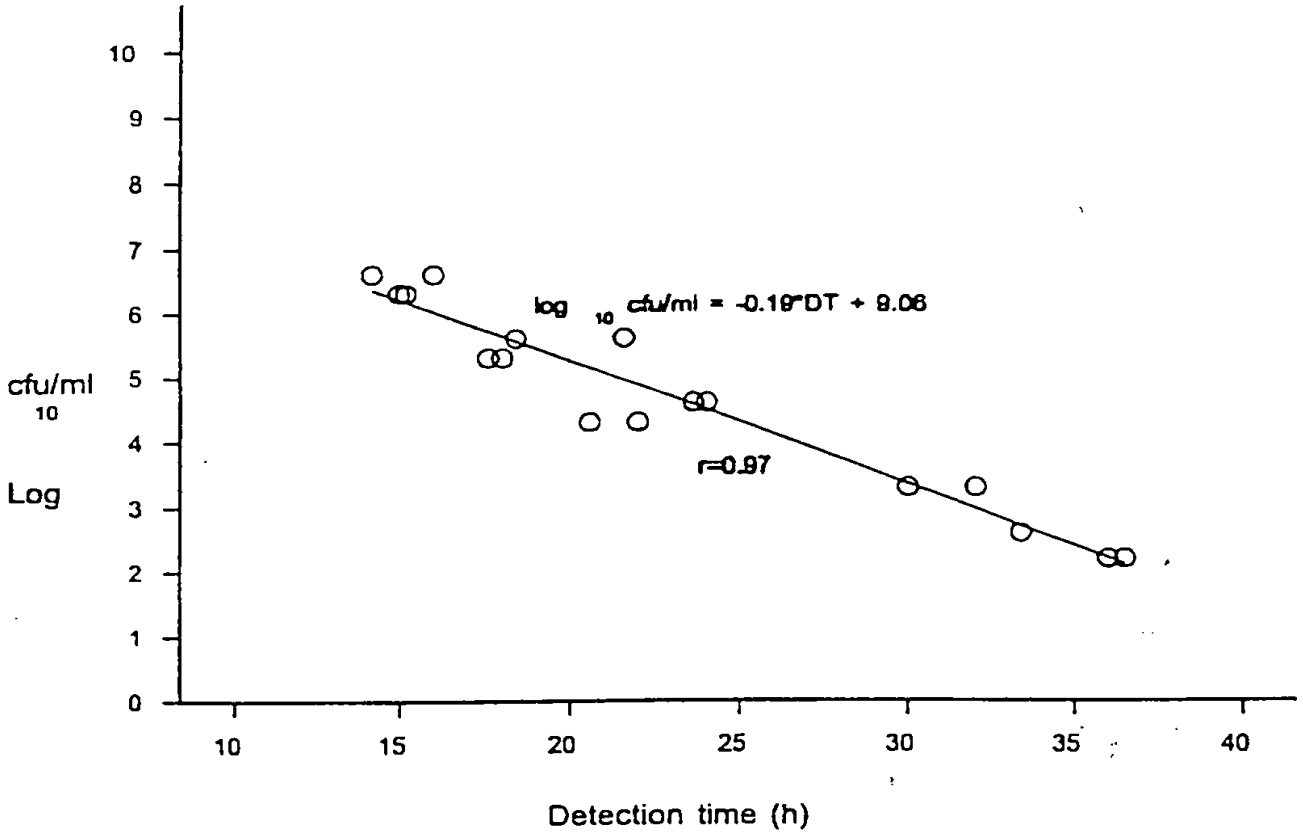

Fig. 7

INTERNATIONAL SEARCH REPORT

International application No.
PCT/DK 97/00283

A. CLASSIFICATION OF SUBJECT MATTER

IPC6: C12N 9/24, C12N 15/56
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC6: C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WPI, CA, BIOSIS, MEDLINE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 9602653 A1 (NOVO NORDISK BIOTECH, INC.), 1 February 1996 (01.02.96), see claims 5-6, 17 --	1-24
X	File WPI, Derwent accession no. 92-111673, NISSIN SHOKUHIN KAISHA LTD: "Alpha-1,3-glucanase gene - obtd. by lighting DNA into vector, transforming it into oral cavity bacteria and secretion of enzyme protein"; & JP,A,4058889, 920225 --	1-24
X	US 4353891 A (BERNHARD GUGGENHEIM EET AL), 12 October 1982 (12.10.82), see column 4, line 6-63 --	17

[X] Further documents are listed in the continuation of Box C. [X] See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" erlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
29 Sept 1997	13 -10- 1997
Name and mailing address of the ISA/ Swedish Patent Office Box 5055, S-102 42 STOCKHOLM Facsimile No. +46 8 666 02 86	Authorized officer Yv nne Siösteen Telephone No. +46 8 782 25 00

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/DK 97/00283

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 5521093 A (YVES LEMOINE ET AL), 28 May 1996 (28.05.96) --	1,5,7,13
A	EP 0327377 A2 (SUNTORY LIMITED), 9 August 1989 (09.08.89) --	1,5,7,13
A	Dialog Information Services, file 155, MEDLINE, Dialog accession no. 07915749, Medline accession no. 94237475, Castelli LA et al: "High-Level secretion of correctly processed beta-lactamase from Saccharomyces cerevisiae using a high-copy-number secretion vector"; & Gene May 3 1994, 142 (1) p113-7 --	1,5,7,13
A	Chemical Abstracts, Volume 112, No 13, 26 March 1990 (26.03.90), (Columbus, Ohio, USA), page 238, THE ABSTRACT No 113593v, CN, 86102686 A,, (matsushiro, aizo) 21 October 1987 (21.10.87) -- --------	1-24

INTERNATIONAL SEARCH REPORT

Information on patent family members

01/09/97

International application No.
PCT/DK 97/00283

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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		EP 0771354 A	07/05/97
		FI 970200 A	17/01/97
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		ZA 9506014 A	22/02/96
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		AT 318815 B	25/11/74
		AU 468059 B	18/12/75
		AU 3474371 A	03/05/73
		BE 774420 A	25/04/72
		CA 996877 A	14/09/76
		CH 571571 A	15/01/76
		DE 2152620 A,B,C	27/04/72
		FR 2110067 A	26/05/72
		GB 1373487 A	13/11/74
		NL 175734 B,C	16/07/84
		NL 7114567 A	28/04/72
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		SE 426650 B,C	07/02/83
		ZA 7106878 A	28/06/72
US 5521093 A	28/05/96	AT 109506 T	15/08/94
		CA 2013239 A	30/09/90
		DE 69011183 D,T	16/03/95
		EP 0396436 A,B	07/11/90
		SE 0396436 T3	
		ES 2060974 T	01/12/94
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		PT 93614 B	30/08/96
EP 0327377 A2	09/08/89	SE 0327377 T3	
		AT 123520 T	15/06/95
		CA 1335966 A	20/06/95
		DE 68922917 D,T	26/10/95
		ES 2072893 T	01/08/95
		JP 1199578 A	10/08/89
		US 5162220 A	10/11/92
		US 5234830 A	10/08/93
CN 86102686 A	21/10/87	NONE	

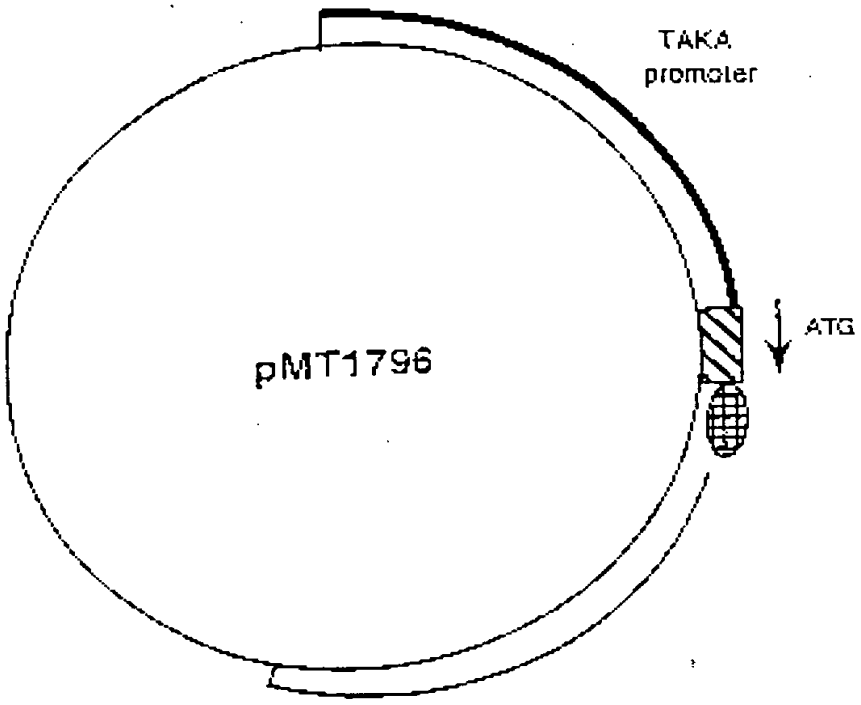


Fig. 1

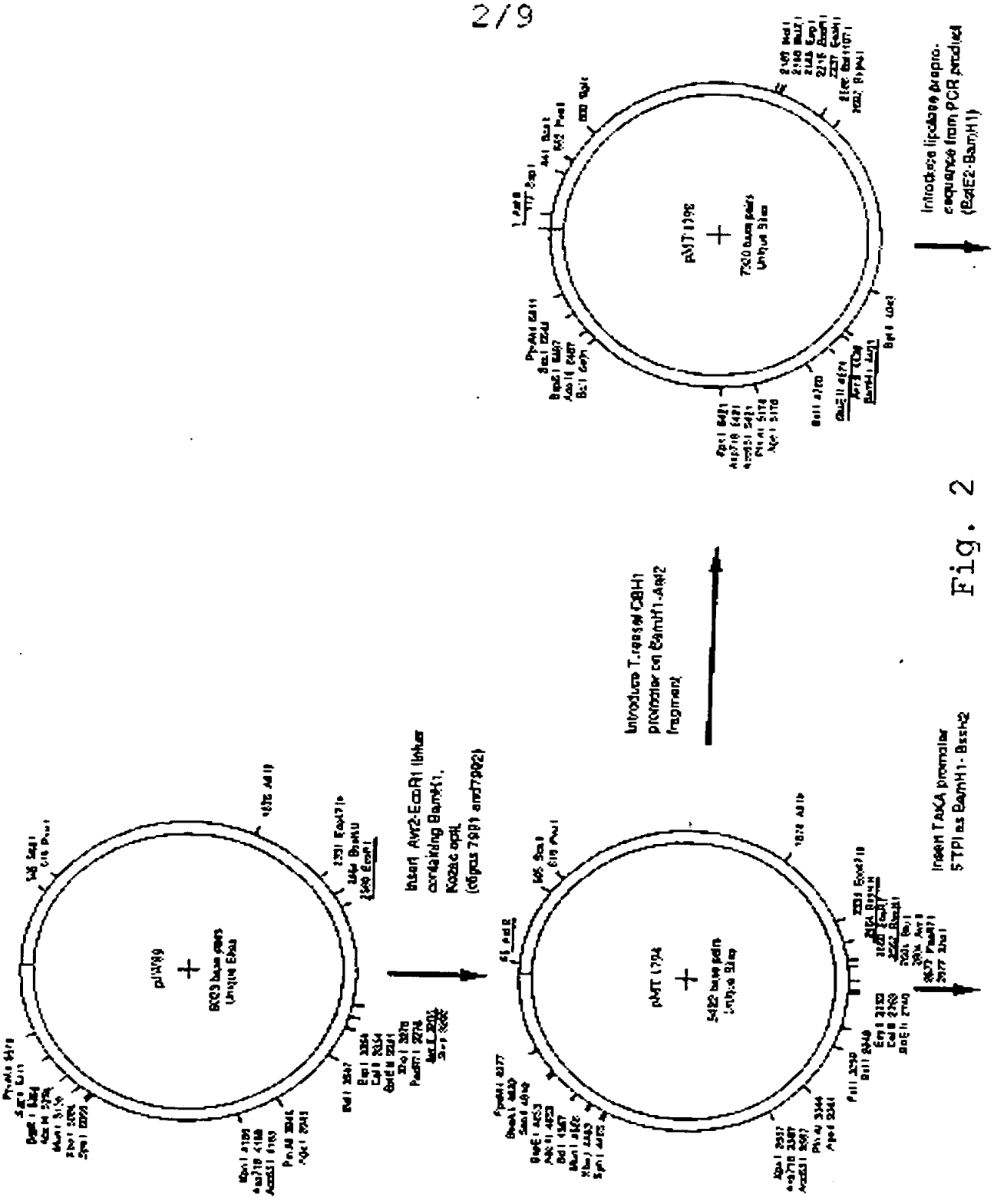


Fig. 2

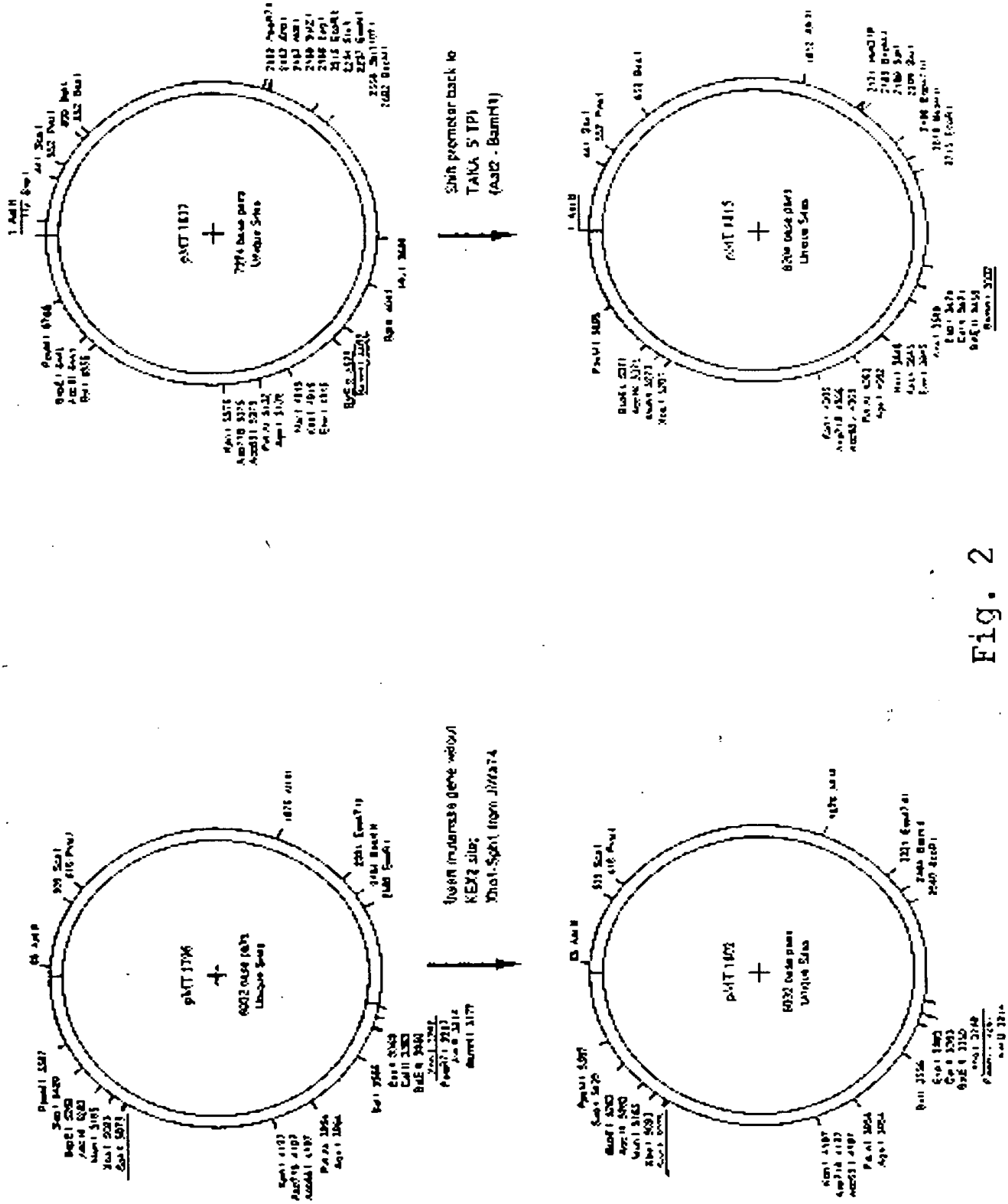


Fig. 2

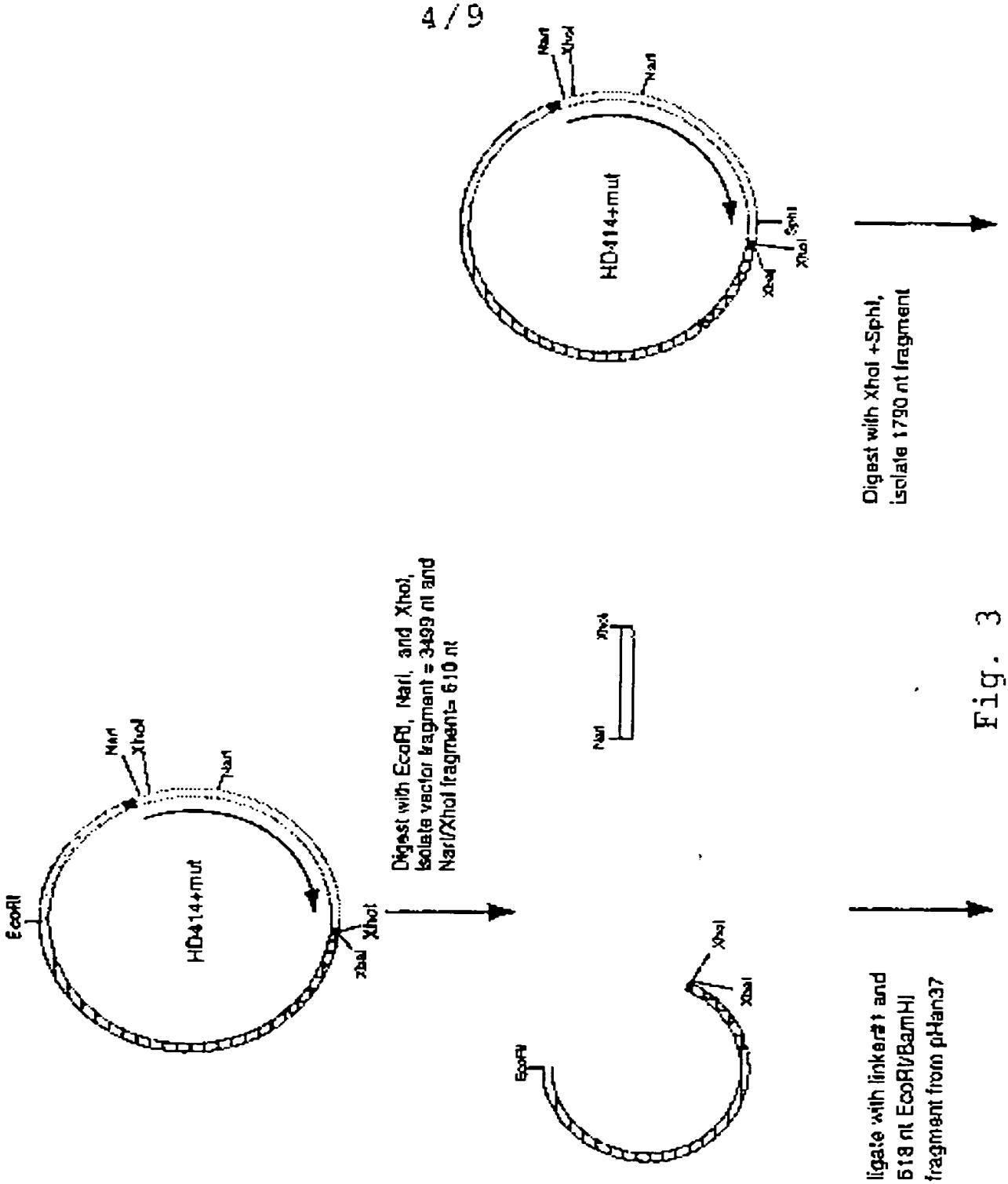

HD414+mut
NarI
XhoI
EcoRI
XbaI
Digest with EcoRI, NarI, and XhoI, isolate vector fragment = 3499 nt and NarI/XhoI fragment= 610 nt
Digest with XhoI +SphI, isolate 1790 nt fragment
ligate with linker#1 and 618 nt EcoRI/BamHI fragment from pHan37

Fig. 3

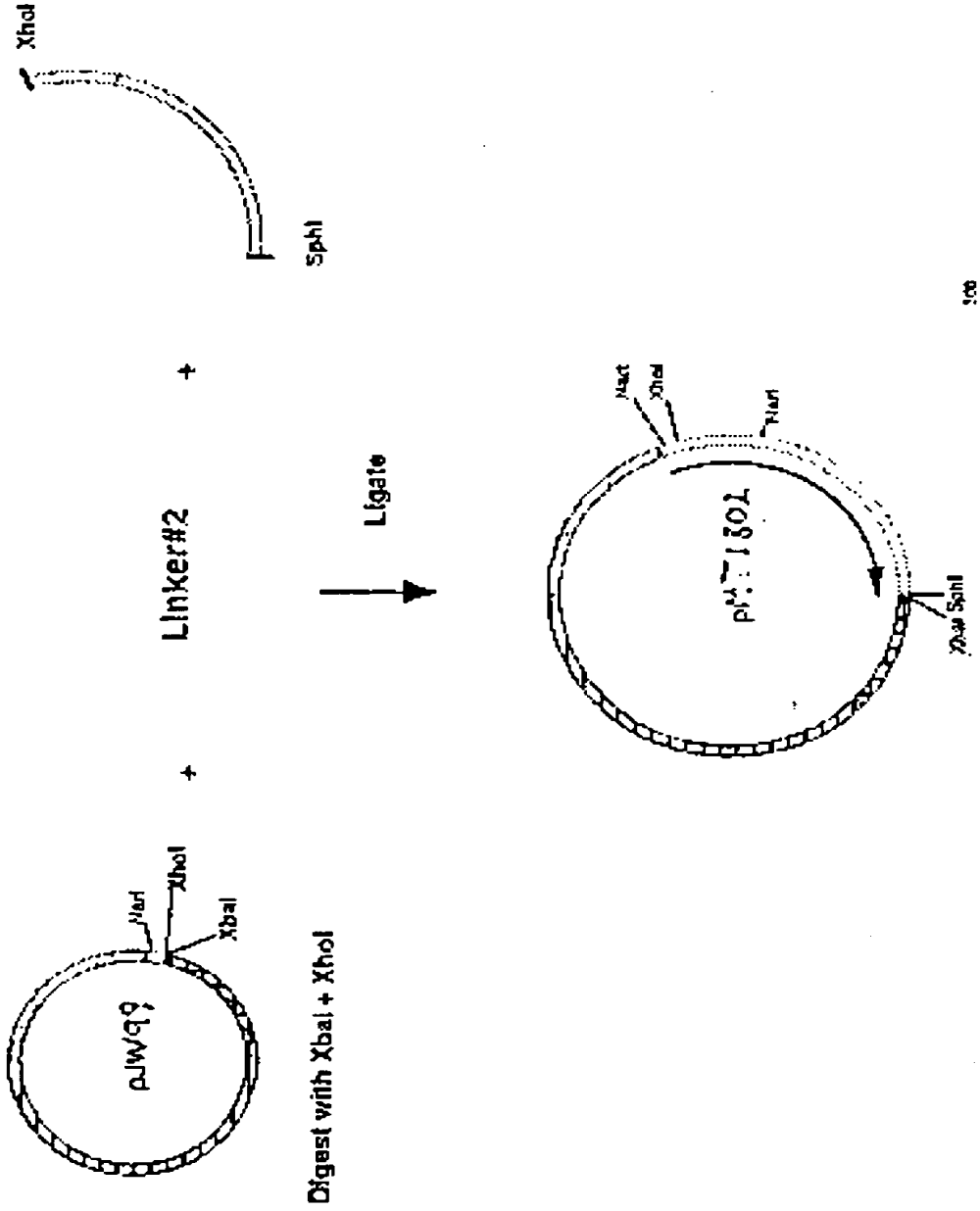


Fig. 3

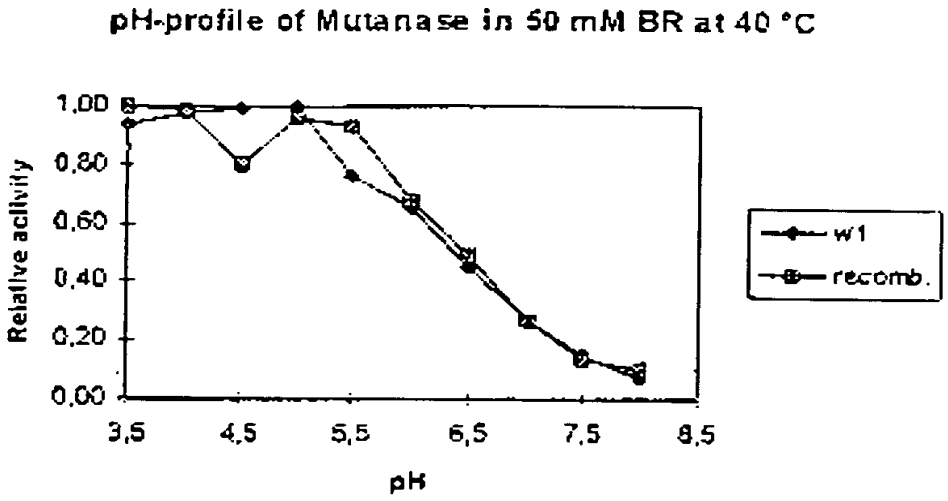
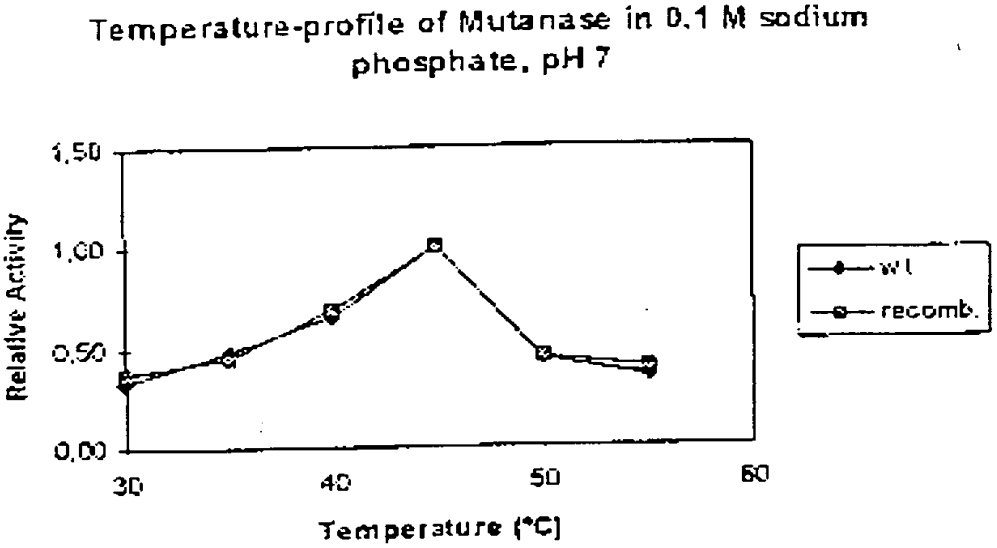

pH-profile of Mutanase in 50 mM BR at 40 °C
Relative activity
1,00
0,80
0,60
0,40
0,20
0,00
3,5
4,5
5,5
6,5
7,5
8,5
pH
w1
recomb.

Fig. 4



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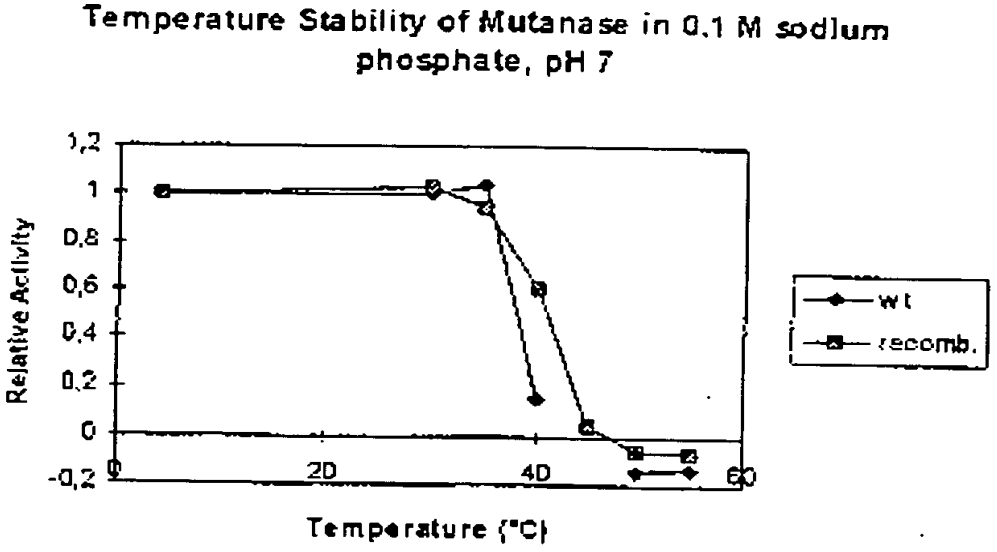

Fig. 6

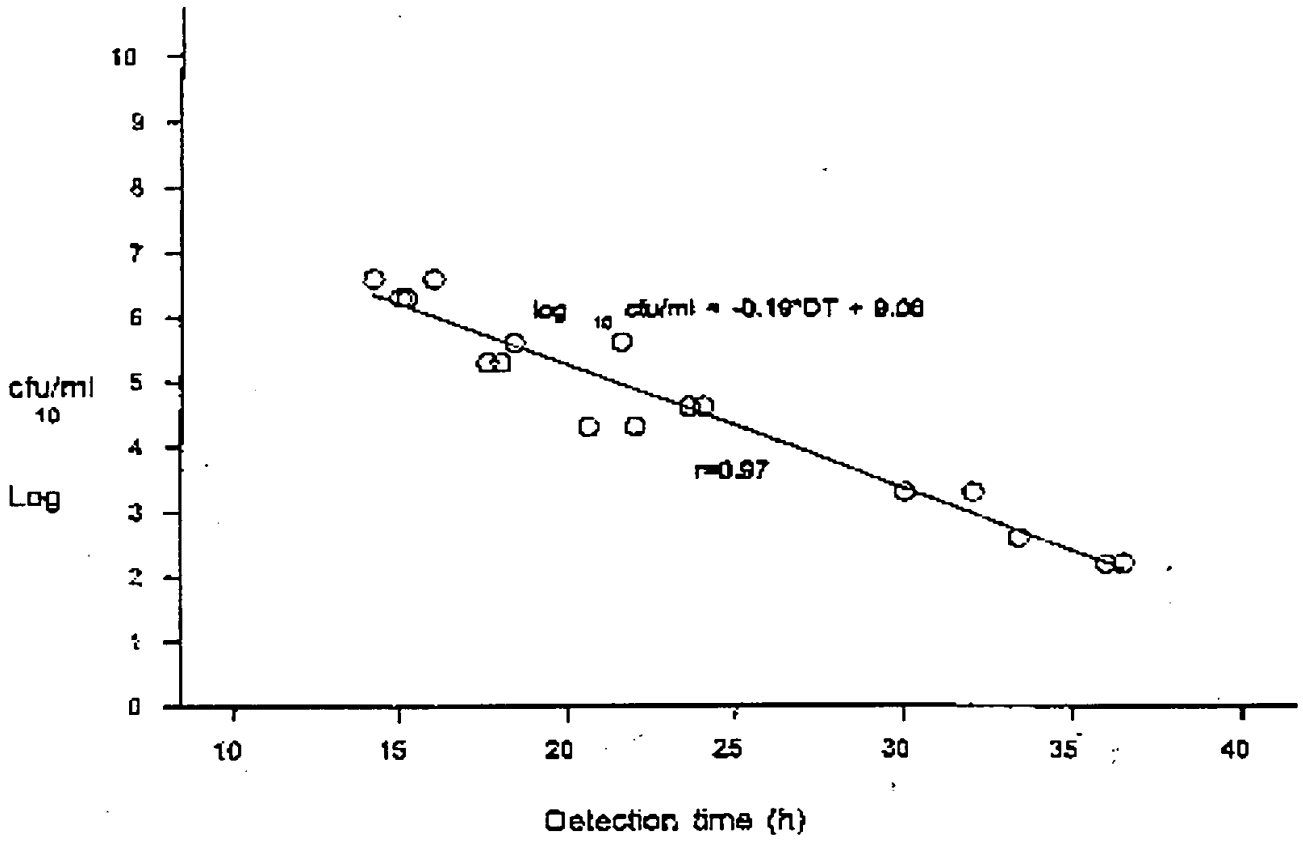

Fig. 7